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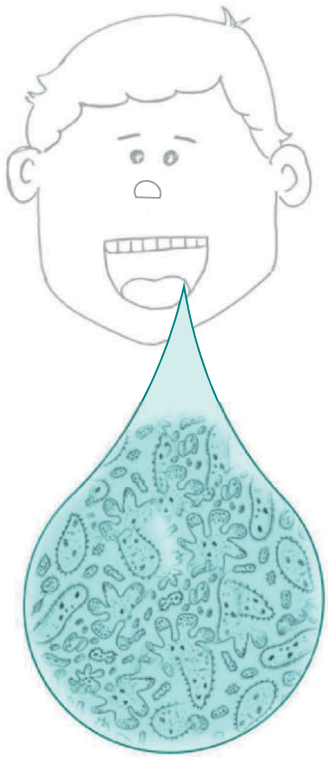

SALIVARY MICROBIOTA – HOW TO MEASURE IT AND ITS ASSOCIATIONS WITH BODY SIZE AND ANTIMICROBIAL USE

SAJAN RAJU

Salivary microbiota - how to measure it and its associations with body size and antimicrobial use

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Folkhälsan Research Center

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Public Health, Faculty of Medicine

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PUBLICATIONS

I. **Raju, S. C**., Lagström, S., Ellonen, P., de Vos, W. M., Eriksson, J. G., Weiderpass, E., & Rounge, T. B. (2018). Reproducibility and repeatability of six high-throughput 16S rDNA sequencing protocols for microbiota profiling. Journal of Microbiological Methods, 147(March), 76–86. https://doi.org/10.1016/j.mimet.2018.03.003

II. **Raju, S. C.,** Lagström, S., Ellonen, P., Vos, W. M. De, Eriksson, J. G., Weiderpass, E., & Rounge, T. B. (2019). Gender-specific associations between saliva microbiota and body size. Frontiers in Microbiology, 10(April), 1–10. https://doi.org/10.3389/fmicb.2019.00767

III. **Raju, S. C**., Viljakainen, H., Figueiredo, R.A.O., Neuvonen, P. J., Eriksson, J.G., Weiderpass, E., Rounge, T.B. Number of antimicrobial drugs prescriptions in the first decade of life influences saliva microbiota diversity and composition. *Submitted manuscript*.

The publications are referred to in the text by their Roman numerals.

ABBREVIATIONS

16S rRNA	16S ribosomal ribonucleic acid
AM	Antimicrobial
ANCOVA	Analysis of co-variance
ANOVA	Analysis of variance
AvoHilmo	Outpatient database
BMI	Body mass index
bp	basepair
DNA	deoxyribonucleic acid
FDR	False discovery rate
fitZig	Zero inflated Gaussian fit
GLM	General linear models
ICC	Intra class correlation coefficient
ii	Internal index
IOTF	International Obesity Task Force
KELA	Social Insurance Institution of Finland
KW-test	Kruskal-Wallis test
NGS	Next generation sequencing
NX-tailed	Nextera tailed
NX-tailed-2S	Nextera tailed 2 step
OTU	Operational Taxonomic Unit
PCoA	Principal component analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance
RDP	Ribosomal database project
S.D	Standard deviation
SCFA	Short chain fatty acid
SILVA	SILVA rRNA database project
THL	National Institute for Health and Welfare
TS-tailed	Truseq tailed
TS-tailed-1S	Truseq tailed 1 step
TS-tailed-2S	Truseq tailed 2 step
V3-V4 region	Variable regions 3 and 4

ABSTRACT

The human microbiota, i.e. the microbes living in or on humans, plays an important role in health and disease. Lifestyle factors, such as diet and physical activity as well as environmental factors, and exposure to antimicrobials (AMs) are likely among the important factors shaping the microbiota. Several studies have reported that the gut microbiota may be associated with overweight and obesity. Fewer studies have investigated associations between body mass index (BMI) and the salivary microbiota. There is a worldwide epidemic of obesity and the number of overweight and obese people reached 1.9 billion in 2016. Overweight and obese children are likely to stay obese into adulthood and tend to develop diseases more frequently and at a younger age. Thus, identification of the associations between microbiota and body size in young individuals are of great importance.

The increased use of AMs rises concern of increasing antibiotic resistance resulting in lack of treatment options for many diseases. Exposure to AMs affects the microbial diversity and composition in the gut microbiota, but less is known about salivary microbiota. Since AM use is frequent in children, it is vital to study its associations with saliva microbiota at this age. The objectives of this doctoral thesis were to develop cost effective protocols to assess the salivary microbiota profiles for large-scale epidemiological studies and, with the new protocol, to determine its association with body size and lifetime antimicrobial use in children.

In this thesis, all in-house 16S amplicon assays produced similar salivary microbiota profiles for the individual samples, i.e. there was no superior protocol. Salivary microbiota profiles of Finnish children were gender-specific in terms of alpha- and beta-diversity and relative abundances of bacteria. A prominent finding was the decrease in the core bacteria in overweight and obese children. Lifetime AM exposure to saliva microbiota showed that Azithromycin use was associated with alpha-diversity in all children, and in girls. Microbiota dissimilarities were significant between children with low, medium and high number of AM user groups in all children with all AMs combined. Similar trend was significant with Azithromycin use, whereas Amoxicillin use affected the dissimilarity only in boys.

This thesis suggests that the saliva microbiota is significantly associated with body size, antimicrobial use and gender in Finnish children. Thus, saliva microbiota profiles open new possibilities to study the potential roles of microbiota in weight development and management in children. In addition, the involuntary consequences of lifetime AM use are a concern and the importance of microbiota in the development of new therapeutic strategies should be emphasized in order to limit the use of AMs wisely. Studies have shown that the saliva microbiota is more resilient and stable than gut microbiota when exposed to antibiotics. Thus, the saliva-based screening of microbial biomarkers in

health surveillance, and the associations with oral and general health status, may be considered feasible, simple, economical and easy to collect with high compliance for all age groups compared to faecal samples. However, further research on metabolic and functional potential of saliva microbiota is needed to fully understand the saliva microbiota – host relationship.

1. INTRODUCTION

Human microbiota research, i.e., studies of the microorganisms that live in or on humans and their role in health and disease, has gained significant interest in the last few years (Bai et al., 2018; Cho and Blaser, 2012; Gao et al., 2018; Hakansson et al., 2011; Thaiss, 2018; Valdes et al., 2018; Wu et al., 2018). It has been proposed that gut microbes are the key to many aspects of human health, including immune (Zhang et al., 2015b), metabolic (Rothschild et al., 2018) and neuro-behavioural traits (Levy et al., 2017). Recoveries from animal models (Fierer et al., 2008; Wiley et al., 2017) and human (Beaumont et al., 2016; Falony et al., 2016; Goodrich et al., 2014b; Sonnenburg and Sonnenburg, 2014) support the role of gut microbiota in health. Dysbiosis is broadly defined as any modification to the composition of resident commensal microbial communities relative to the microbial community found in healthy individuals (Petersen and Round, 2014). In the past decade, researchers have reported dysbiotic microbiota in human and animal models with inflammatory bowel diseases (IBD) (Frank et al., 2007), diabetes (Karlsson et al., 2013), asthma (Abrahamsson et al., 2014), allergies and even autism (Parracho et al., 2005) and many other diseases (Carding et al., 2015; Degruttola et al., 2016; Kho and Lal, 2018). Dysbiosis of the human gut microbiota may alter pathogenic mechanisms, which are associated with the development of obesity and insulin resistance (Musso et al., 2010) and with body size (Turnbaugh et al., 2006). Little is known about the salivary microbiota and body size, although the oral cavity possesses the second most diverse microbial community in the body (Kilian et al., 2016).

The oral cavity is an open system and more exposed to the external environment, being the major gateway for microorganisms into the human body (Dewhirst et al., 2010). The oral cavity possesses around 600–700 common species, however, only around 68% of them have been cultivated using aerobic and anaerobic microbiological methods (Chen et al., 2010; Dewhirst et al., 2010). Lifestyle factors, such as diet, physical activity, environmental factors and socio-economic status are likely important factors shaping the oral microbiota and affecting the microbial composition of saliva (Belstrom et al., 2016; Li et al., 2014b). The salivary microbiota is more resilient than the faecal microbiota toward exposure to antibiotics (Zaura et al., 2015), and short-term hospitalization does not alter its composition (Cabral et al., 2017). The short-term ecological consequences of antibiotics on the gut microbiota are fairly well-established (Ferrer et al., 2017; Korpela et al., 2016), but less is known about effects of lifetime antimicrobial (AM) use on the salivary microbiota.

The prevalence of obesity is increasing worldwide and the number of overweight and obese people reached 1.9 billion in 2016 (NCD Risk Factor Collaboration, 2017). Health consequences of obesity can be seen already in childhood and become more prevalent in adulthood (Turta and Rautava, 2016). Overweight and obese children are likely to

stay obese into adulthood and tend to develop diseases frequently and at a younger age. Moreover, with the increased use of antibiotics, we are currently facing the highest threat of antibiotic resistance worldwide (Leong et al., 2018). The human microbiota has a range of effects on the hosts physiological processes and behaviour (Festi et al., 2014; Jumpertz et al., 2011). Thus, investigating the associations between the microbiota and body size and antimicrobials in young individuals are of great importance. Microbiological studies using saliva rely on well-developed protocols, exploiting culture-independent next generation sequencing (NGS) technologies. A challenge using NGS is to ensure high reproducibility and repeatability of the method. The aims of the doctoral thesis were to develop an accurate methodology to assess the salivary microbiota profiles for large-scale epidemiological studies and, with the new method, to determine its associations with body size and lifetime AM use in children.

2. REVIEW OF LITERATURE

2.1 Human microbiome

The human microbiome likely plays an important role in health and well-being. The term microbiome was coined in 2001 and defined as "*ecological community of commensal, symbiotic and pathogenic microorganisms"* which include archaea, bacteria, viruses, and microscopic eukaryotes, including fungi (Lederberg and McCray, 2001; Peterson et al., 2009). Collectively, all these microbes can be referred to as microbiota, while a group of these organisms and their environments such as the cells, genomes and metabolites of the host are referred to as microbiome (Whiteside et al., 2015; Young, 2017). Human microbiota research has been focusing on bacteria due to their clinical significance and the technical challenges to characterise microbes other than bacteria. It has been estimated that microbial cells in the human body (human microbiome) outnumber human cells by a factor of 10 or more (Bäckhed et al., 2005), but the recent estimate suggests that the ratio of human cells to microbial cells is approximately 1:1.3 (Sender et al., 2016). Individual body habitats typically harbour dozens of bacterial phyla and hundreds of bacterial phylotypes. Phylotype defined as observed similarity used to classify a DNA sequence or group of sequences by their evolutionary relationship (Moreira and López-García, 2014). The number of bacteria may differ dramatically between individuals, across body habitats, and within an individual body habitat over time; however, the majority of body habitats are dominated by just a few bacterial phyla (Fierer et al., 2012) (Figure 1). Humans are not an autonomous organism; together with microbiome we form a 'superorganism' or holobiont with microbial community playing a significant role in human physiology and health (Bordenstein and Theis, 2015) (Figure 2). The mutually beneficial relationship between the host and microbiota has provided essential contributions to human health e.g. by producing metabolites and vitamins and in return are allowed to occupy a nutrient rich environment (Salonen and de Vos, 2014)**.** The microbiota is also beneficial in the maturation of the human immune system (Nylund et al., 2014). Among all the human microbiome from different body habitats, the gut microbiota is the most studied and focused upon by researchers; there were nearly 12,900 articles published on this topic between the years 2012 to 2017 (Cani, 2018). Since invasive procedures required to collect samples from the gastrointestinal (GI) tract, most of the studies use stool samples to get an overview of the gut microbiota (Cani, 2018). It comprises of bacteria, archaea, eukaryotes and viruses, and 1000 bacterial species in the gut are dominated by the two phyla, Firmicutes and Bacteroidetes (Figure 1) (Eckburg et al., 2005). This microbial community with millions of genes provides wide range of additional metabolic potential to complement human genome functions (Li et al., 2014a).

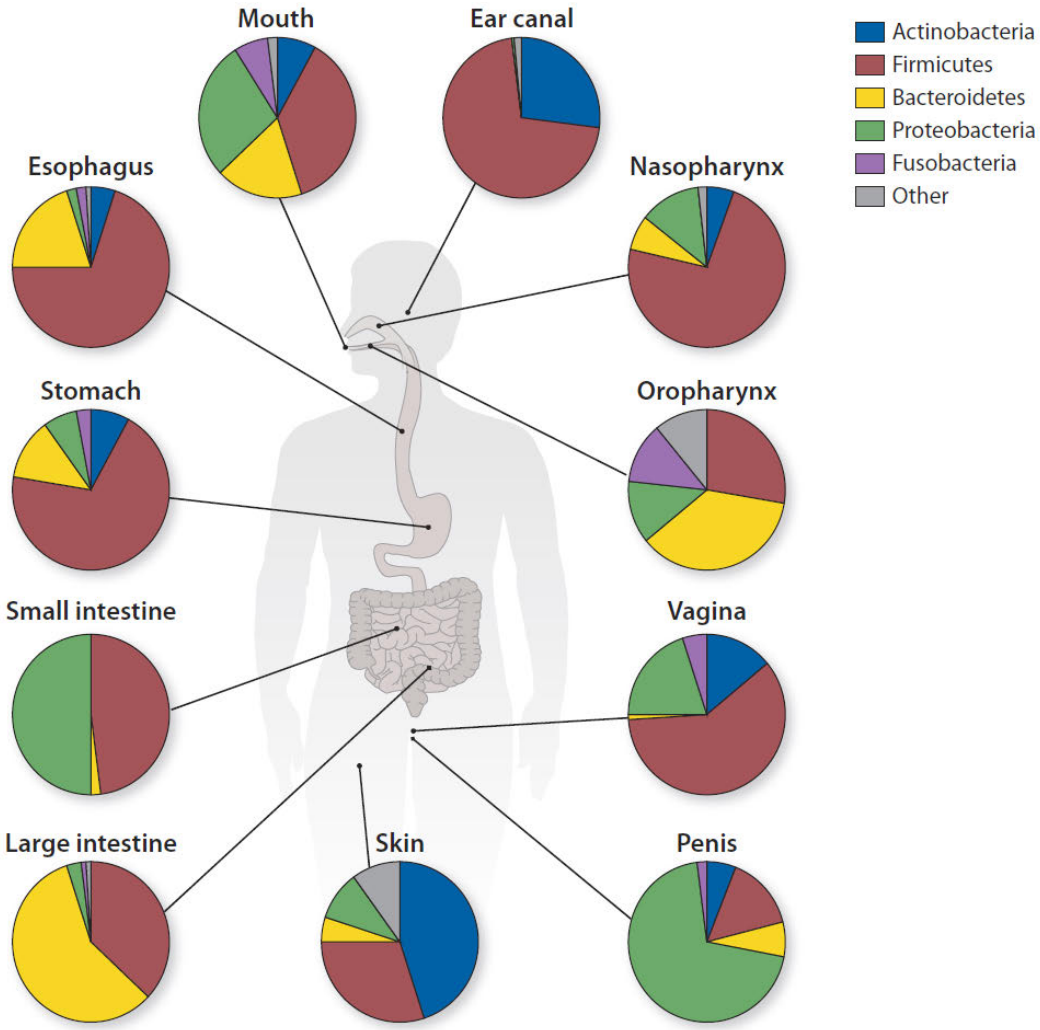

Figure 1: Composition of dominant bacterial phyla in various body habitats. Figure modified from Fierer et al., 2012.

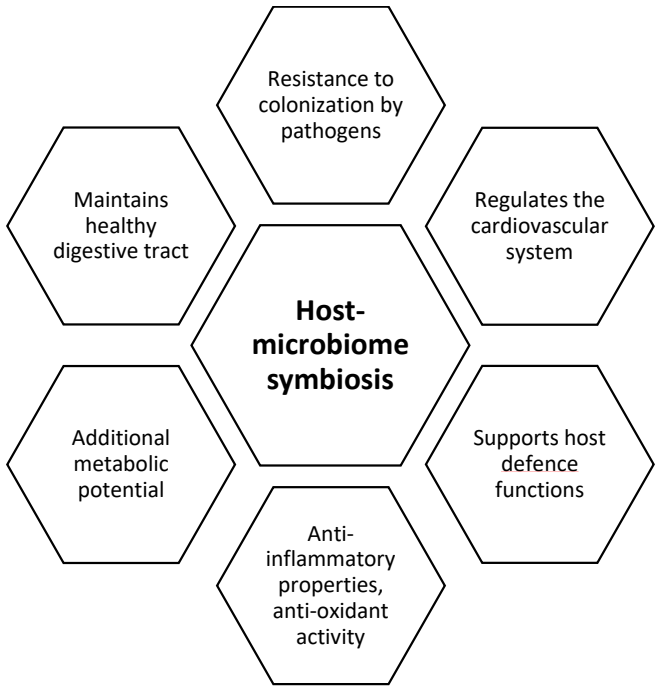

Figure 2: The positive effects of the microbiome on human. Figure modified from Kilian et al., 2016

Unlike the human genome, the gut microbiome can be vertically transmitted and fixed; it can also be modified by early life events, diet and exposure to antibiotics affecting the composition, stability and function of the gut ecosystem. At the lower level of genera and species, the bacterial composition of the microbiota shows inter-individual variation, i.e. each individual possesses a unique microbial fingerprint. The microbial fingerprint is stable over time and resilient, but not to perturbations like antibiotic exposure (Leong et al., 2018).

2.1.1 Oral microbiota

The oral cavity is highly exposed to the environment and is the major gateway for microorganisms into the human body. Microorganisms enter the body through food and air, and are then either mixed with saliva on the way to the intestinal tract, or left behind to colonize the oral cavity (Dewhirst et al., 2010). The oral cavity comprises several distinct microbial habitats, such as teeth, gingival sulcus, attached gingiva, tongue, cheek, lip, hard palate, and soft palate. The human oral microbiome defined as all the microorganisms that are present on or in the human oral cavity and its contiguous extensions (such as tonsils, pharynx, esophagus, Eustachian tube, middle ear, trachea, lungs, nasal passages, and sinuses) (Dewhirst et al., 2010). Different oral habitats and tissues are colonized by distinct microbial communities (Aas et al., 2005; Mager et al., 2003). The oral cavity also harbors microbes as planktonic cells or incorporated into

biofilms. Oral biofilms, a unique survival strategy of microbes, are present on all intra-oral surfaces. Biofilm helps microbes to adapt to environmental change by altering their gene expression patterns which can protect the microbes from disinfectant agents or antibiotics (Berger et al., 2018). A balanced oral microbiome assist in maintaining oral health and symbiosis. Dysfunction of salivary gland often disturbs the oral microbiome which leads to oral and systemic diseases associated with it (Lynge Pedersen and Belstrøm, 2019).

2.1.2 Saliva microbiota

The microorganisms in the human saliva (the saliva microbiome) have been associated with diseases of both the oral cavity and general health (Krishnan et al., 2017). Bacteria in the saliva are especially shed from adhering microbial communities on various intraoral surfaces, including tooth surfaces, gingival crevices, tongue dorsum, and buccal mucosa (Takeshita et al., 2016). Saliva microbiota profiles enable the detection of various oral infectious diseases and upper respiratory infections (Wong, 2012). The salivary microbiota is more resilient than the gut microbiota toward exposure to antibiotics, irrespective of antibiotics taken (Zaura et al., 2015), and to short-term hospitalization (Cabral et al., 2017). The saliva microbiota remains highly stable over several years, even when changes in diet and oral hygiene may occur (Stahringer et al., 2012). Microbes in the oral cavity are the etiological agents for a number of infectious diseases such as caries, periodontitis, endodontic infections, alveolar osteitis and tonsillitis (Duran-Pinedo and Frias-Lopez, 2015).

2.1.3 Saliva as a diagnostic bio-fluid

Saliva is an aqueous, transparent biological fluid containing a complex mixture produced by secretions from the salivary glands, combined with bacteria of the oral cavity, desquamated cells and food debris (Humphrey and Williamson, 2001; Nieuw Amerongen and Veerman, 2002; Pappa et al., 2019). Saliva is generated by acinus cells within the salivary glands and released into the oral cavity via small ducts (Tiwari, 2011; Yoshizawa et al., 2013). Saliva plays a key role in keeping a 'healthy mouth'. Evidently, a reduction in saliva flow increases the risk of dental caries. It lubricates the oral surfaces and helps in reducing demineralization of damaging acids from biofilms, clearing food and promoting remineralization (Marsh et al., 2016). Saliva is an ideal diagnostic fluid useful for studies in vulnerable patient populations such as neonates and children as it is an easy, simple, painless and non-invasive method for sampling (Figure 3) (Ranger and Grunau, 2014; Schafer et al., 2014). Saliva harbors wide range of structurally and functionally complex host molecules (proteins and glycoproteins) which are primary nutrients for the resident microbiota (Marsh et al., 2016). Saliva is rich in biomarkers for oral and systemic disorders, such as antibodies, DNA, RNA, metabolites, cytokines,

enzymes and hormones like cortisol (Giannobile et al., 2011; Kononen et al., 2007; Yoshizawa et al., 2013).

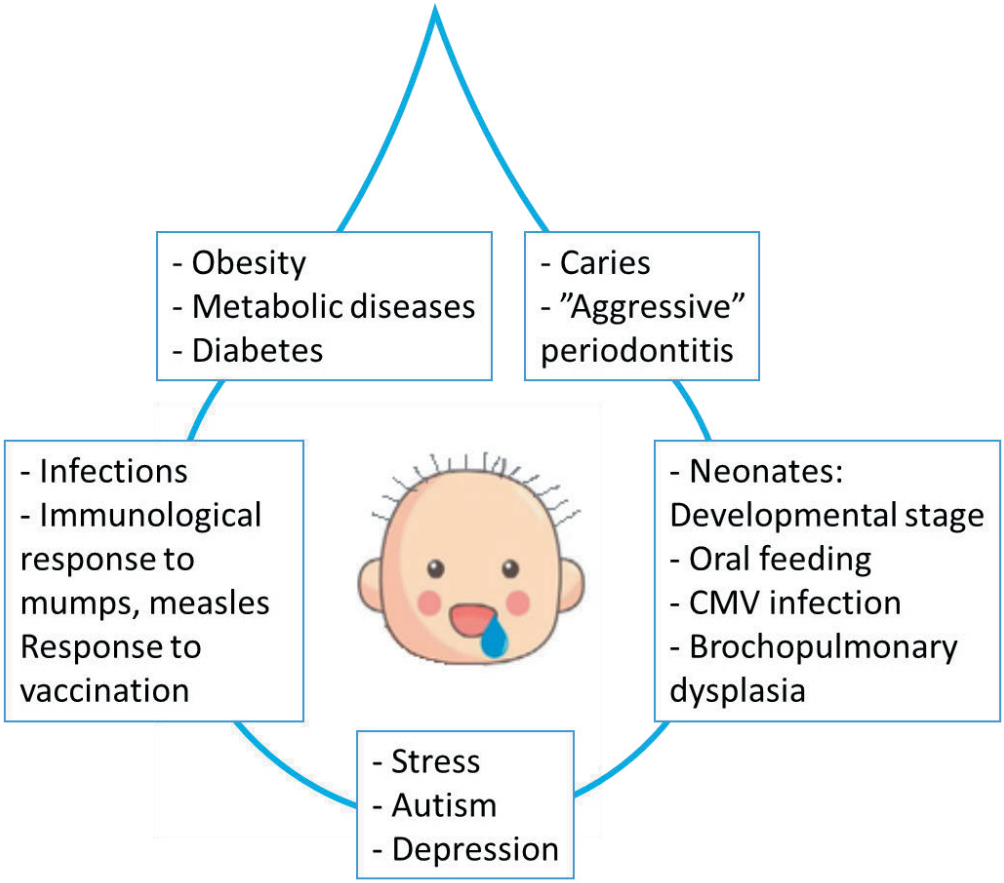

Figure 3: Applications of the saliva as a diagnostic fluid in paediatrics. Figure modified from Pappa et al., 2019.

2.2 Microbiota profiling

Interest in studying associations of the human microbiota with human health status using the 16S rRNA gene, a phylogenetically informative housekeeping genetic marker, has rapidly increased (Lane et al., 1985; Tringe and Hugenholtz, 2008; Zheng et al., 2015). Taxonomic investigation in microbiota studies is based on amplification of hypervariable regions of the 16S rRNA gene sequences for several reasons: the 16S rRNA gene is present in all archaea and bacteria, it has both conserved and species-specific variable regions, it is extensively studied and comprehensive databases (SILVA, RDP and Greengenes) exist (Tseng et al., 2013). Cultivation-independent molecular methods have validated different estimates and identified approximately 600 species or phylotypes in the oral cavity using 16S rRNA gene sequencing techniques

(Dewhirst et al., 2010; Paster et al., 2001). Precise microbiota profiling depends heavily on the hypervariable region of the 16S rRNA gene selected and primers used, and the amplification of non-representative genomic regions may lead to taxonomic resolution bias (Wen et al., 2017; Zheng et al., 2015). Development of next generation sequencing (NGS) technologies and the application of barcode indexing help to sequence a large number of samples simultaneously (Andersson et al., 2008; de Muinck et al., 2017; Hamady et al., 2008). However, reproducibility and repeatability of the microbiota profiling remain challenging (Ding and Schloss, 2014). The 16S rRNA V3-V4 hypervariable region is widely used in various microbiota studies, although other hypervariable regions have also been used (Belstrøm et al., 2016; Fadrosh et al., 2014; Janem et al., 2017; Zheng et al., 2015). Introduction of dual indexing and its application to the sequencing protocols helped to yield a greater utilization of available sequencing capacity (Kozich et al., 2013). Recent advancements and reduced prices have directed researchers to use the latest technologies while abandoning the old ones; however, the evaluation of new methodology is crucial and should be performed before conducting microbiome studies (Sinclair et al., 2015).

2.3 Microbiota and obesity

2.3.1 Obesity

Obesity is "condition of abnormal or excessive fat accumulation in adipose tissue, to the extent that health may be impaired" (World Health Organization, 2000). There is a worldwide epidemic of obesity and the number of overweight and obese people globally has almost tripled since 1975 (World Health Organization, 2018). In 2016, 1.9 billion (39%) of adults aged 18 years and above were overweight, and 650 million (13%) of these were obese (World Health Organization, 2018). A measure of obesity is the body mass index (BMI), a person's weight (in kilograms) divided by the square of his or her height (in metres). For adolescents under 18 years of age, BMI cut-offs used are age- and gender-specific (Cole and Lobstein, 2012).

The mechanism behind the obesity believed to be disorder with multiple causes, i.e: genetics, environmental factors, life styles and socio-cultural factors (Figure 4). In general, overweight and obesity are identified to be the results of increased calorie and fat intake (Sahoo et al., 2015).

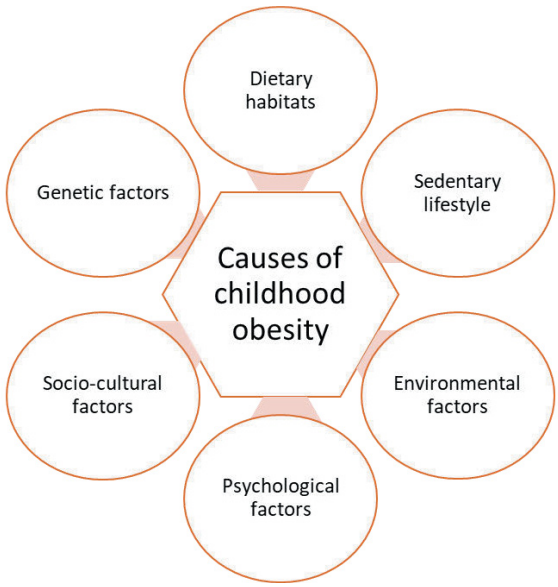

Figure 4: Causes of childhood obesity. Figure modified from Sahoo et al., 2015.

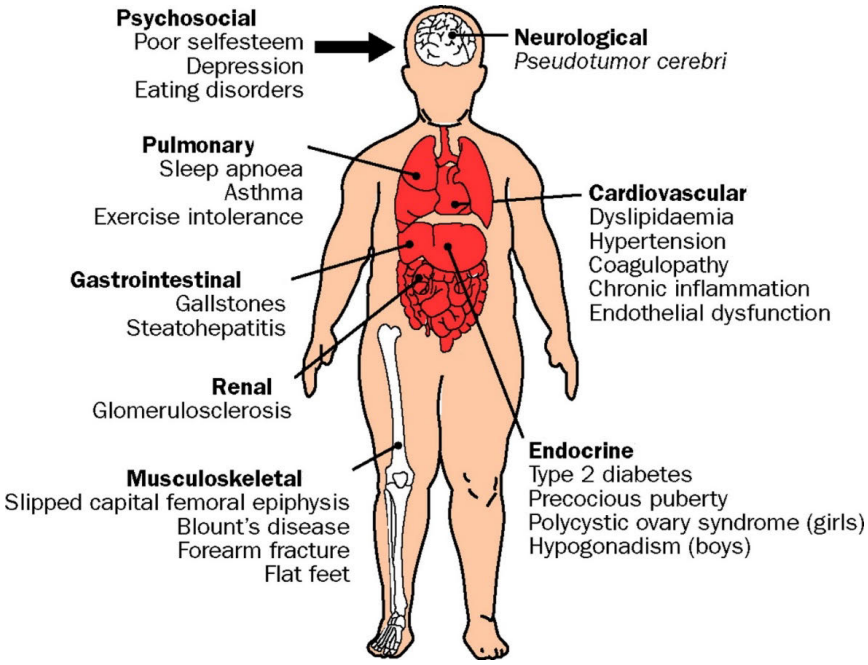

Figure 5: Consequences of childhood obesity. Figure modified from Ebbeling et al., 2002.

According to the World Health Organization, around 340 million children and adolescents aged 5-19 years were overweight or obese. Overweight and obese children are likely to stay obese into adulthood (Juonala et al., 2019; Turta and Rautava, 2016;

Woo et al., 2019), unless remedial measures are taken to tackle it, and they are inclined to develop diseases more frequently and at a younger age than normal weight children. The health consequences of obesity (Figure 5), which include type 2 diabetes mellitus, non-alcoholic fatty liver disease, and ischemic cardiovascular disease may be initiated already in childhood and become more prevalent in adulthood (Despres and Lemieux, 2006; Turta and Rautava, 2016). Other health consequences are related to neurological (Balcer et al., 1999), cardiovascular (Ford et al., 2001; Srinivasan et al., 2002; Tounian et al., 2001), endocrine (Ebbeling et al., 2002; Kaplowitz et al., 2001; Lewy et al., 2001), musculoskeletal (Dowling et al., 2001; Goulding et al., 2001; Loder and Skopelja, 2011), renal (Adelman et al., 2001), gastrointestinal (Friesen and Roberts, 1989; Strauss et al., 2000), pulmonary diseases (Figueroa-Muñoz et al., 2001; Redline et al., 1999; Reybrouck et al., 1997) and psychosocial consequences (Balcer et al., 1999; Davison and Birch, 2001; Erickson et al., 2000; Field et al., 1999; Strauss, 2000). In addition, obesity is associated with lower life expectancy, poor quality of life, work disability and healthcare costs (Collaboration et al., 2016; Fontaine and Barofsky, 2001; van Duijvenbode et al., 2009). The prevalence of obesity in adults has extensively increased during 1978-2017 in Finland (Borodulin et al., 2018). Nowadays every fourth man and woman is obese in Finland. Also, the prevalence of severe obesity has increased and, one of 20 men and one of 10 women are severely obese. If the current scenario continues in Finland, every third adult is expected to be obese by 2028 (Borodulin et al., 2018).

The prevalence of overweight and obesity in children and adolescents is also increasing globally, with a threefold increase in Finland (NCD Risk Factor Collaboration, 2017). According to the International Obesity Task Force (IOTF) using BMI cut-off values for adolescents (Cole and Lobstein, 2012), 19% of 2-16-year-old Finnish children and adolescents were classified as overweight, and 5% of boys and 4% of girls as obese in 2014–2015 (Mäki et al., 2017). According to a recent report from the National Institute for Health and Welfare (THL) based on outpatient health care register data (AvoHilmo), 21% of girls and 38% of boys aged 7-12 years were overweight or obese (Lundqvist and Jääskeläinen, 2019).

2.3.2 Gut microbiota and obesity

Associations between the gut microbiota and obesity have been shown in both animal models and humans (Parekh et al., 2015; Sze and Schloss, 2016). For example, the ratio of Firmicutes to Bacteroidetes (F/B ratio) was lower in obese mice than in lean mice (Ley et al., 2005). Human studies have shown that obese individuals had lower alpha-diversity than lean individuals (Turnbaugh et al., 2009) and that obesity in adults and children is associated with different profiles of gut microbiota (Castaner et al., 2018; Goodrich et al., 2014a, 2014b; Hu et al., 2015; Ley et al., 2006; Nakayama et al., 2015; Turnbaugh et al., 2009). A recent study reported a higher abundance of family Tissierellaceae and genus *Blautia,* and lower abundance of *Methanobrevibacter* in the

gut microbiota of obese vs. normal weight adults (Org et al., 2017). From the limited research focused on obesity in children and adolescents and their gut microbiota; *Bacteroides* and *Prevotella* were significantly associated with the adolescent BMI (Hu et al., 2015), abundance of nine bacteria was associated with overweight and obesity in Mexican children (Murugesan et al., 2015) and an altered gut microbiota characterized by high abundance of Firmicutes and lower Bacteroidetes was associated with obesity (Riva et al., 2017). Bacterial dysbiosis (imbalance) has been linked to the development of obesity, either through direct effect on the gut or indirect regulation on distal organs and systems (Clarke et al., 2014; Leong et al., 2018).

Intestinal bacteria are capable of breaking down the indigestible polysaccharides (fibre) and resistant starch to short chain fatty acids (SCFA) to provide 80-200 kcal/d energy, and dysbiosis may alter the energy harvest in the gut (Jumpertz et al., 2011). SCFAs also modulate the production of gut hormones which directly influence satiety (Festi et al., 2014). Intestinal bacteria have a key role in bile acid metabolism that can have a strong effect on host energy metabolism. Dysbiosis may fail to de-conjugate toxic primary bile acids produced by the host, by bacterial bile-salt hydrolases, leading to reduced numbers of Lactobacilli and Bifidobacteria considered protective against obesity (Kurdi et al., 2006).

2.3.3 Saliva microbiota and obesity

Oral bacteria have been associated with a number of oral (Gao et al., 2018), non-oral and systemic diseases (He et al., 2015; Krishnan et al., 2017) and cancer (Mager et al., 2005) (Table 1)**.** Dysbiosis in the gut microbiome is associated with obesity and its co-morbidities (Turnbaugh et al., 2006), however, little attention has been paid to saliva microbiota and obesity. Goodson et al first postulated the association of the oral microbiota with obesity in 2009 (Goodson et al., 2009).

Table 1: Summary of findings from studies on salivary microbiota with non-oral diseases. Table adapted from (Acharya et al., 2017a).

Diseases	References	Key findings (in disease)
Auto-immune diseases		
Celiac disease	Tian et al., 2017	High abundance- *Lactobacilli* (both in refractory and treated celiac disease cases)
Celiac disease	Siragusa et al., 2014	High abundance - Lachnospiraceae, Gemellaceae, *Streptococcus sanguinis*, *Porphyromonas* sp., *Prevotella nanceiensis*, *Rothia*, SR1, Low abundance- Bacilli, *Selenomonas* and *Actinomyces oris* Lower alpha-diversity (richness)
Sjögren's syndrome	Sandhya et al., 2015	High abundance - *Capnocytophaga*, *Dialister*, *Fusobacterium*, *Helicobacter*, *Streptococcus*, *Veillonella* spp. and lower *Pseudomonas* spp.
Sjögren's syndrome	Li et al., 2016	Low abundance- Proteobacteria Lower alpha-diversity (richness)
Behçet's disease	Coit et al., 2016	High abundance - *Haemophilus parainfluenzae* Low abundance - *Alloprevotella rava* and *Leptotrichia*
Behçet's disease	Seoudi et al., 2015	High abundance - *Bifidobacterium dentium*, *Prevotella histicola* Low abundance - *Campylobacter concisus*, *Clostridiales* spp., *Fusobacterium periodonticum*, *Gemella sanguinis*, *Neisseria* spp., and *Oribacterium sinus* levels in disease
Rheumatoid arthritis	Zhang et al., 2015c, 2015a	High abundance - *Lactobacillus salivarius* levels Low abundance - *Haemophilus* spp.
Inflammatory bowel disease	Said et al., 2014	High abundance of Bacteroidetes and Low abundance - Proteobacteria, salivary immunological biomarkers correlated significantly with several dominant genera e.g.; *Prevotella* with interleukin-1 beta
Immunoglobulin A nephropathy	Piccolo et al., 2015	Low abundance - Firmicutes to Proteobacteria ratio, Health-associated taxa - *Gemella haemolysans*, *Granulicatella adiacens*, *Veillonella parvula* and many *Prevotella* spp.
Cancer		
Pancreatic cancer	Fan et al., 2018	High abundance - *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans* associated positively Fusobacteria, *Leptotrichia* associated negatively with pancreatic cancer risk.
Pancreatic cancer	Torres et al., 2015	High abundance - *Leptotrichia* to *Porphyromonas* ratio Low abundance - *Neisseria* and *Aggregatibacter*
Pancreatic cancer and chronic pancreatitis	Farrell et al., 2012	High abundance of *Granulicatella adiacens*, Low abundance - *Neisseria elongata*, and *Streptococcus mitis*

Esophageal cancer	Chen et al., 2015	High abundance - *Prevotella*, *Streptococcus* and *Porphyromonas*, Low abundance - *Lautropia, Bulleidia, Catonella, Corynebacterium, Moryella, Peptococcus, Cardiobacterium* Lower alpha-diversity
Nasopharyngeal carcinoma	Xu et al., 2014	High abundance – Firmicutes, *Neisseria*, *Leptotrichia*, *Pseudomonas* Low abundance - Proteobacteria, *Streptococcus*
Colorectal cancer	Flemer et al., 2018	Differentially abundant in CRC - *Streptococcus* and *Prevotella*
Genetic disorders		
Fanconi's anemia & history of transplant	Furquim et al., 2017	High abundance - Firmicutes, *Veillonella*, *Streptococcus*, *Haemophilus* in patients with graft versus host disease. Higher *Streptococcus*, *Haemophilus*, *Aggregatibacter*, *Selenomonas*, *Capnocytophaga*, *Corynebacterium* with severe oral mucositis
Cystic fibrosis (pediatric)	Zemanick et al., 2015	Lower alpha-diversity & greater divergence in composition from Lower airway microbiome (beta-diversity) was associated.
Parkinson's disease (PD)	Pereira et al., 2017	PD patients and control subjects had differences in beta-diversity Differences in abundances of individual bacterial taxa
Immunodeficiency states		
HIV	Noguera-Julian et al., 2017	High abundance - *Neisseria* in HIV-positive and High abundance - *Treponema* in HIV-positive with severe periodontal disease.
HIV-AIDS	Zhang et al., 2015a, 2015c	High abundance - *Porphyromonas, Treponema, Eubacterium* in AIDS patients with periodontitis.
HIV (well-controlled)	Kistler et al., 2015	*Haemophilus parainfluenzae* associated positively & Streptococcus mitis (HOT473) negatively with disease, no major differences in community composition with disease. *Haemophilus parainfluenzae, Actinomyces* positively associated and *Alloprevotella tannerae, Eubacterium yurii* negatively associated with CD4 cell counts
HIV (well-controlled, pediatric)	Goldberg et al., 2015	No significant differences in community configuration (beta-diversity) or alpha-diversity between disease and health
HIV (before and after Highly active retroviral therapy)	Li et al., 2014c	High abundance - Bacteroidetes, Low abundance - Actinobacteria, Fusobacteria , *Fusobacterium, Campylobacter, Leptotrichia* Lower alpha-diversity with disease.
Transplant associated immunosuppression	Diaz et al., 2013	High abundance - Gammaproteobacteria; *Klebsiella pneumoniae, Pseudomonas flurosccns, Acinetobacter* spp., *Vibrio* spp., *Enterobacteriaceae* spp., *Enterococcus faecalis* in transplant patients.
Radiation associated immunodeficiency	Schuurhuis et al., 2016	High abundance of opportunistic species; enteric rods, *Staphylococcus*, *Candida* after radiation therapy (with and without chemotherapy both)

Metabolic disorders		
High interleukin-1 beta levels	Acharya et al., 2017b	Beta-diversity & *Prevotella* to *Bacteroides* ratio, associated with interleukin-1 beta levels. High abundance - *Proteobacteria* spp, *Aggregatibacter, Halomonas, Propionivibrio, Paracoccus, Mannhemia, Anaerobacillus, Micrococcus*, Bradyrhizobiaceae, Caulobacteraceae were indicators of high interleukin-1 beta.
Diabetes mellitus-Type 2 & Obesity (pediatric)	Janem et al., 2017	Diabetes mellitus showed lower levels of *Lautropia, Cardiobacterium, Corynebacterium*
Diabetes mellitus-Type 2	Kampoo et al., 2014	High abundance - *Streptococcus* spp., *Lactobacillus* spp. associated with high caries incidence in diabetics
High salivary glucose levels	Goodson et al., 2017	High abundance - Firmicutes, *Parvimonas micra*, Low abundance - Bacteroidetes, *Prevotella* spp. predicted high salivary glucose.
Stroke	Boaden et al., 2017	No association of salivary microbiota configuration and oral health status
Polycystic ovary syndrome	Lindheim et al., 2016	Low abundance – Actinobacteria
Atherosclerosis	Fåk et al., 2015	High abundance - *Parvimonas, Capnocytophaga, Catonella, Lactobacillus* associated with serum biomarkers.
Hepatic encephalopathy	Bajaj et al., 2015	High abundance - opportunistic taxa, Prevotellaceae, Fusobacteriaceae and Enterococcaceae, Enterobacteriaceae, Low abundance - resident taxa, Erysipelothricaceae in disease. Enterobacteriaceae correlated negatively with antiinflammatory cytokine Interleukin-10.
Other		
Antibiotic treated Low birth weight neonate	Costello et al., 2013	High abundance - *Pseudomonas aeruginosa*, *Ureaplasma parvum*, a novel *Mycoplasma* sp. were found after antibiotic treatment in a low birth weight infant

The reports on the association of the oral microbiome with obesity are inconsistent. In a study including 87 adolescents, a six-fold increase of *Campylobacter rectus* and *Neisseria mucosa* was found in subgingival biofilms of obese subjects in comparison with lean (Zeigler et al., 2012). According to an Italian study including 28 obese and 28 normal weight adults Firmicutes and Actinobacteria were more abundant in saliva microbiota of obese individuals, while Proteobacteria and Fusobacteria dominated in normal weight individuals (Piombino et al., 2014). In contrast, a Danish study including 292 adults failed to show an association between BMI and saliva microbiota (Belstrøm et al., 2014). A periodontal pathogen *Tannerella forsythia* has been reported to be positively correlated with BMI in periodontally healthy adults (Haffajee and Socransky, 2009; Stahringer et al., 2012). In a recent Japanese study focusing on saliva microbiome, a higher phylogenetic diversity was observed in individuals with obesity; however, the effects of periodontal disease might have confounded the results (Takeshita et al., 2016). A recent study showed that obesity was associated with the saliva microbiota composition (Tam et al., 2018).

2.4 Microbiota and antimicrobials (AMs)

2.4.1 Use of antimicrobials

Antimicrobials, hailed as a 'miracle drug', have revolutionized the field of medicine because they effectively treat infectious bacterial diseases (Zaffiri et al., 2012). AMs are a group of agents that share the common aim of reducing the growth or killing microorganism. AMs are made synthetically or from moulds, and are absorbed into the body to kill the bacteria (bactericidal) or to prevent the bacterial multiplication (bacteriostatic) (Nankervis et al., 2016). AMs are the most commonly used drugs in paediatric patients and have helped to reduce the morbidity and mortality associated with bacterial infections in children (Principi and Esposito, 2016; Zhang et al., 2013). However, the increased use of AMs can cause unwanted problems such as development of AM resistance and AM-induced gut microbiota dysbiosis (Principi and Esposito, 2016). Paediatric populations are more prone to use AM, in 2011, 40% of children under 16-year-old have used antibiotics at least once in Finland (Virta, 2012).

2.4.2 Gut microbiota and antimicrobials

Exposure to AMs has a high impact on all microbiomes compared to other environmental factors (Modi et al., 2014). The gut microbiota in adults is strongly affected by AMs administration, and it is non-resilient to repeated AM use (Dethlefsen and Relman, 2011). The short-term ecological consequences of AMs on the gut microbiota are fairly well-established (Ferrer et al., 2017; Korpela et al., 2016), but less is known about the effects of lifetime AM use on saliva microbiota. Amoxicillin

is a commonly used drug and prescribed in dentistry for chemoprophylaxis of infective endocarditis; and in a study of 4 to 5-year-old children, amoxicillin-resistant oral bacteria have been observed (Lazarevic et al., 2013b; Ready et al., 2004). The use of AM including amoxicillin, erythromycin, josamycin and tetracycline can increase the number of antibiotic-resistant bacteria in the oral cavity (Harrison et al., 1985; Ready, 2002; Ready et al., 2004; Sefton, 1999; Woodman et al., 1985). The effect of a 6-week intravenous AM therapy in humans found significant changes in oral and faecal bacterial communities (Abeles et al., 2015). The gut microbiota is prone to large shifts in the diversity and composition with several perturbations like diet, exercise and antimicrobials (Cabral et al., 2017). The instability of the gut microbiota due to the antimicrobial exposure could make it difficult in drawing the conclusion of microbiome shifts and their relevance to human health in clinical settings (Cabral et al., 2017). AM agent clindamycin cause dysbiosis higher than ciprofloxacin in gut (Rashid et al., 2015), and it takes 1 to 12 months to normalise the microbiota (Francino, 2016).

2.4.3 Salivary microbiota and antimicrobials

Salivary microbiota seems to be consistent between individuals and is considerably less diverse in comparison to the gut microbiota (Zaura et al., 2015). A study that compared the salivary and faecal microbiota after AM use demonstrated that saliva microbiota is more resilient than the gut microbiota (Zaura et al., 2015). A single dose of 2g amoxicillin can cause dysbiosis in oral cavity and induce selection of resistant strains, genus Streptococci with reduced susceptibility to amoxicillin was significantly increased (Chardin et al., 2009; Khalil et al., 2016). The prevalence of penicillin-resistant *Fusobacterium nucleatum* in infants has been shown (Nyfors et al., 2003), due to increase in β-lactamase producing species with the use of antimicrobial agents during the first year of life (Nyfors et al., 1999). The effect of single AM dose and short-term AM therapy on saliva microbiota in adults have been shown, where single dose of most commonly used AMs causes minor and short-term changes in saliva microbiota (Abeles et al., 2015; Lazarevic et al., 2012; Zaura et al., 2015). The saliva microbiota of adults treated with azithromycin fully regained its original status within eight weeks, while the effects were longer lasting in the gut (Abeles et al., 2016). Thus using saliva microbiota, which shows more temporal stability, may help to understand different long-term antimicrobial treatments or perturbations effect on the human microbiota (Cabral et al., 2017).

3. AIMS OF THE DOCTORAL THESIS

The objectives of this thesis were to develop cost effective protocols to characterize saliva microbiota profiles and to study the association of the saliva microbiota with body size, and lifetime antimicrobial use in children. The specific aims of the studies in this thesis were:

1. To identify the reproducibility and repeatability of the saliva microbiota profiles produced with simplified in-house 16S amplicon assays by comparing triplicate samples (I).

2. To discover the association between the saliva microbiota and body size in a large-scale study of Finnish children (II).

3. To discover the association between lifetime antimicrobial use and the saliva microbiota diversity and composition in Finnish children (III).

4. MATERIALS AND METHODS

4.1 Ethical aspects

The Coordinating Ethics Committee of the Hospital District of Helsinki and Uusimaa has approved the Fin-HIT study protocol (169/13/03/00/10). Participants and one of their parents have given written informed consent, which allows us to integrate the national health register data as a part of the research material (Figure 7). Participants had right to withdraw their consent whenever they wish to.

4.2 Study population

All the subjects included in the studies II and III were from the "The Finnish Health in Teens study — Fin-HIT — cohort (Table 2 and 3), which includes approximately 11,000 Finnish children in the age 9-14 years and their legal guardians (Figueiredo et al., 2018). The cohort participants were from densely populated regions of Finland, i.e; Uusimaa, Varsinais-Suomi, Häme, Pirkanmaa, Keski-Suomi, Pohjois-Savo and Pohjois-Pohjanmaa regions. Geographically, a large part of Finland was represented in the study, including 44 municipalities and some of Finland's largest cities: Helsinki, Turku, Espoo, Oulu, Jyväskylä, Tampere and Kuopio. Recruitment of participants and baseline data collection was performed during 2011-2014 (Figure 7).

Figure 7: Schematic presentation of the Fin-HIT cohort data collection at school.

Table 2: Characteristics of children included in study II

Body sizes	Boys	Girls	Total
Underweight	48	77	125
Normal weight	307	353	660
Overweight	52	44	96
Obese	10	9	19
Total	417	483	900

Table 3: Characteristics of children included in study III

Characteristics	Children	
	N	%
Age, yrs		
11	227	27.1
12	610	72.9
Gender		
Boy	388	46.4
Girl	449	53.6
Language		
Finnish	711	84.9
Swedish	89	10.6
Other	37	4.4

4.2.1 Saliva samples

Saliva samples in triplicates from four volunteers were collected for study **I** (Figure 6). The saliva (unstimulated) samples were collected in Oragene-DNA (OG-500) self-collection kits (DNA Genotek Inc., Canada) (**I, II & III**). The samples were mixed with a stabilizing reagent within the collection tubes per manufacturer's instructions by participants, and stored at room temperature (Figure 8).

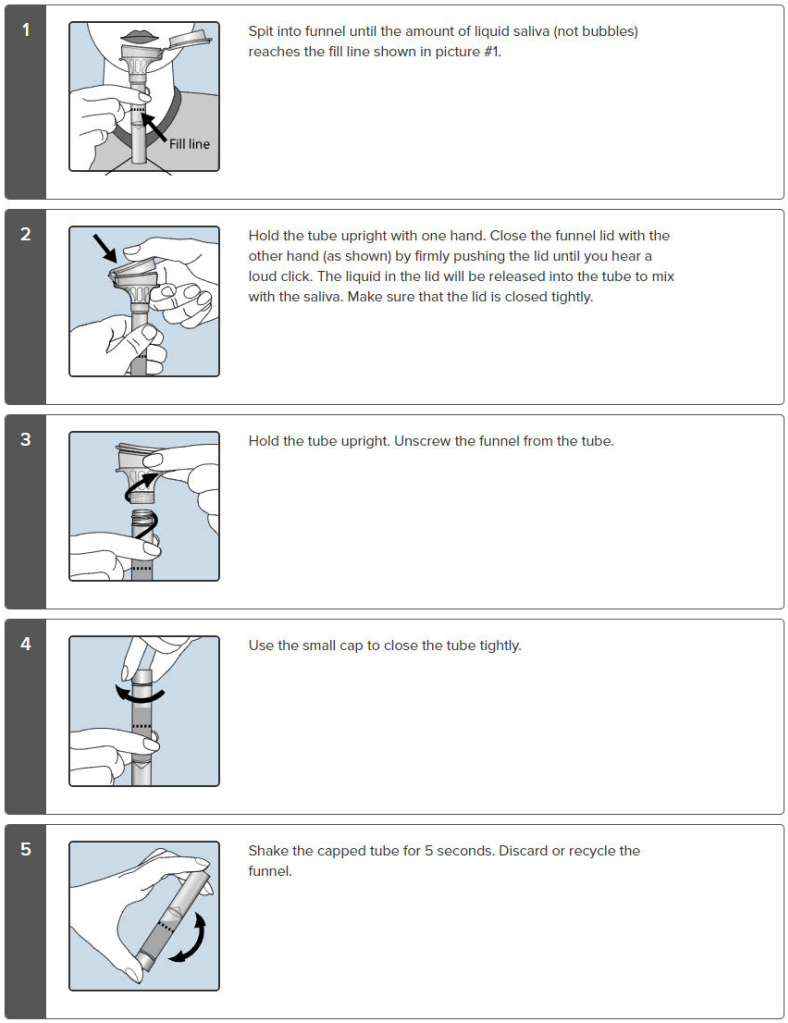

Figure 8: Saliva collection instruction from the Oragene-DNA (OG-500) self-collection kits (DNA Genotek Inc., Canada). https://www.dnagenotek.com/ROW/support/collection-instructions/oragene-dna/OG-500andOG-600.html

4.2.2 BMI groups/ Body size

The fieldworkers followed a standardized protocol to measure anthropometric measures: height, weight and waist circumference for all children. BMI was calculated as weight (kg)/height $(m)^2$ and the children were classified as underweight, normal weight, overweight or obese (BMI categories) according to age- and sex-specific cut-offs suggested by International Obesity Task Force (IOTF) (Cole and Lobstein, 2012). BMI categories were used in the study II.

4.3 Cohort data collection

4.3.1 Demographics/questionnaire data

During the cohort data collection, the participants filled in self-administered web questionnaire on lifestyle factors, mental health, adolescent growth environment, provided a saliva sample, and had their anthropometric measures measured in a standardized way at school (Figueiredo et al., 2018; Sarkkola et al., 2016). Information on subjects' lifestyle and health behaviour included diet, meal patterns, physical activity, parents' attitudes toward physical exercise, screen time, TV-programmes watched, bullying, and sleep patterns.

4.3.2 Register data

Additional information on the participants was obtained from the national health registers: (a) the Population Information System at the Population Register Centre (https://eevertti.vrk.fi/etusivu), which contains information on home address and mother tongue; (b) the Social Insurance Institution of Finland, (http://www.kela.fi/), which includes information on medical prescriptions, purchases of prescribed drugs and patient spending on these drugs; and (c) Statistics Finland providing information on occupational status, wages and cause of death and (d) the National Institute for Health and Welfare (THL) providing information of Medical Birth Register, the Care Register for Health Care and the Register of Primary Health Care Visits, and information on diseases, pregnancy, new-born health, diagnoses, etc. (Figueiredo et al., 2018).

4.3.3 Medical prescription/reimbursement data

In Finland, all the antimicrobial (AM) agents for systemic use are available by prescription and sold solely in registered pharmacies. The Drug Prescription Register held by the Social Insurance Institution of Finland (KELA, www.kela.fi) register drug purchases based on prescriptions. Information on drug purchase dates and pharmaceuticals of Fin-HIT cohort participants were linked to the study database. Information on all systemic antimicrobials were extracted. The information on antimicrobials administered in hospitals were not collected. The purchases of antimicrobials is considered to reflect the use of AM of interested for the study. In study **III**, the AM prescriptions used from birth to participation date were restricted in the analysis (Table 3). Among all the AMs; Amoxicillin (ATC-code: J01CA04), Azithromycin (J01FA10), Amoxicillin + enzyme inhibitor (J01CR02) and Phenoxymethylpenicillin (J01CE02) were the four most prescribed AMs to the participants.

4.4 Molecular biology methods

4.4.1 16S rRNA sequencing

All of the studies in this thesis followed the same DNA extraction protocol. A protocol with an intensive lysis step combining the mechanical and chemical lysis using a cocktail of lysozyme and mechanical disruption of microbial cells using bead-beating was employed. Fifty ml lysozyme (10 mg/ml, Sigma-Aldrich), 6 ml mutanolysin (25 KU/ml, Sigma-Aldrich), and 3 ml lysostaphin (4000 U/ml, Sigma-Aldrich) were added to a 500 µl aliquot of cell suspension followed by incubation at 37 ºC for 1 h. Subsequently, 600 mg of 0.1 mm-diameter zirconia/silica beads (BioSpec, Bartlesville, OK) were added to the lysate and the microbial cells were mechanically disrupted using Mini-BeadBeater-96 (BioSpec, Bartlesville, OK) at 2100 rpm for 1 min (Yuan et al., 2012). After lysis, total DNA was extracted using cmg-1035 saliva kit, and Chemagic MSM1 nucleic acid extraction robot (PerkinElmer). Lysis step helps to disintegrate the microbial cells. The desired region of the 16S rRNA gene, the V3-V4 region was amplified using Polymerase chain reaction (PCR).

4.4.2 PCR amplification

PCR amplification and sequencing libraries were prepared according to in-house 16S rRNA gene-based PCR amplification protocols. All protocols used 16S primers (S-D-Bact-0341-b-S-17: 5’ CCTACGGGNGGCWGCAG ’3 and S-D-Bact-0785-a-A-21: 5’ GACTACHVGGGTATCTAATCC 3’) targeting the V3-V4 region as reported previously (Klindworth et al., 2013). In the study I, the 16S primers were modified by adding the Illumina Truseq (TS) and Nextera (NX) adapter sequences to the 5’-ends. Amplification was done using primers with and without incorporated internal index (denoted as ii). Two sets of index primers carrying Illumina grafting P5/P7 sequence were used: in-house index primers with Truseq adapter sequence and Illumina Nextera i5/i7 adapters. All oligonucleotides (except Illumina Nextera i5/i7 adapters) were synthesized by Sigma-Aldrich (St. Louis, MO, USA). Barcodes used to sequence large set samples in one pool, which is economic.

4.4.2.1 TS-tailed 1-step amplification

TS-tailed 1-step PCR amplification was employed in a part of study I and all of the studies II & III. Amplification was performed with 16S rRNA gene-based primer carrying Truseq adapter and Truseq index primer. Separate reactions were done using 16S rRNA gene-based primers with and without incorporated 6-base internal index. Here after this protocol denoted as TS-tailed-1S.

4.4.2.2 TS-tailed 2-step amplification

TS-tailed 2-step amplification protocol was employed only in a part of the study I. Amplification was performed with 16S rRNA gene-based primer carrying Truseq adapter. Separate reactions were done using 16S rRNA gene-based primers with and without incorporated internal index. Following PCR amplification, samples were purified and an additional PCR step was employed to add index sequences to the PCR product. Here after this protocol denoted as TS-tailed-2S.

4.4.2.3 NX-tailed 2-step amplification

NX-tailed 2-step amplification protocol was employed only in a part of the study I. Amplification was performed with 16S rRNA gene-based primers carrying Nextera adapters. Separate reactions were done using 16S primers with and without incorporated internal index. Following PCR amplification, samples were purified and an additional PCR step was needed to add index sequences to the PCR product. Amplification was performed according to Illumina Nextera protocol to amplify tagmented DNA. Here after this protocol denoted as NX-tailed-2S.

4.4.3 Pooling, purification and quantification

Following PCR amplifications, libraries were pooled in equal volumes. Library pool was purified twice with Agencourt® AMPure® XP (Beckman Coulter, Brea, CA, USA) Quantification and quality control of DNA sample pool was analysed on Agilent 2100 Bioanalyzer.

4.4.4 Sequencing

Sequencing of PCR amplicons was performed using the Illumina MiSeq (study I) and Illumina HiSeq 1500 (studies II & III) instrument (Illumina, Inc., San Diego, CA, USA). Samples from study I were sequenced as 251x 2 bp paired-end reads and samples from the studies II & III with 271x 2 bp paired-end reads. DNA extracted from nine blank samples, two water samples and nine control saliva samples (in which 5 samples are replicates of sample 4c) using the above mentioned protocol and amplified with TS-tailed 1S protocol without internal index, and sequencing performed (271 x 2 bp) using the Illumina HiSeq instrument.

4.5 Bioinformatics analyses

Sequencing quality, index trimming and length filtering was carried out and sequences were processed using MiSeq_SOP in mothur pipeline (Version v.1.35.1) (Schloss et al., 2009) and sequences were aligned to ribosomal reference database arb-SILVA (Version V119) (Quast et al., 2012). High quality data was ensured for analysis by removing

sequence reads containing ambiguous bases, homopolymers > 8 bp, more than one mismatch in the primer sequence, less than 10 base pair assembly overlap, or less than default quality score in mothur. Assembled sequences with > 460 bp length, Chimeric sequences (Edgar et al., 2011) and singletons were removed from the analysis. The high-quality assembled sequences were aligned to the SILVA 16S rRNA database and clustered into operational taxonomic units (OTUs) at a cut-off value > 98% sequence similarity. OTUs were classified using the SILVA bacteria taxonomy reference. OTU was normalized by subsampling to a threshold of 500 and 2000 in study **II** and **II** respectively. Alpha-diversity (Shannon and inverse Simpson index) was calculated per sample. Beta-diversity using the Bray Curtis dissimilarity indices was calculated between the samples/body size, and gender in the study **I**, and between AM groups in study **III**. Sequencing depths were categorized into three groups: low <10,000, medium <100,000, high >100,000 sequences and used as a covariate in the statistical models in studies **II** & **III**.

4.6 Statistical analyses

In study **I**, the statistical analyses were conducted with R program (Version 3.4.1) for windows (//www.r-project.org/) and with SPSS (Version 22). Kruskal-Wallis (KW-test) test was performed on the alpha-diversity indices to evaluate the significant differences between the microbial diversity and the (TS-tailed and NX-tailed) methods used. Principal coordinate analysis (PCoA) plotted for beta-diversity was defined with Bray-Curtis distance without normalizing the data using phyloseq R-package Ver 1.22.3 (McMurdie and Holmes, 2014). Intraclass correlation coefficients (ICC) to measure the reproducibility, stability, and accuracy or neutrality of different protocol developed. Six metrics included relative abundances of four major phyla and two alpha-diversity indices used for ICC analysis.

In study **II**, all statistical analyses were conducted with R program (Version 3.4.2) for windows. All participants were classified into four groups according to standard BMI cut-offs for children (Cole and Lobstein, 2012). KW-test was used to evaluate the significant difference in distribution of alpha-diversity measures across the body size groups, and gender. Interactions between gender and body size in the model were also examined. Differences in bacterial community structure between body size groups were tested using permutational analysis of variance (PERMANOVA) test with 999 permutations in Phyloseq R-package. Differentially abundant bacteria were identified at the taxonomic levels of Order, Genus and OTUs using the zero-inflated Gaussian model (*fitZig*), implemented in the metagenomeSeq package-version 1.20.1 (Paulson, 2014; Paulson et al., 2013). Differences in bacterial abundance between a) normal weight vs. underweight, b) normal weight vs. overweight, c) normal weight vs. obese, d) normal weight vs. overweight + obese and e) boy vs. girl were tested. The analyses were adjusted for the sequence depth and gender, and the gender model was adjusted for

sequence depth only. P-values were calculated and adjusted by the false discovery rate (FDR).

In study **III**, all statistical analyses were conducted with R (Version 3.4.2) using the stats4 (Version 3.4.2) vegan (version 2.5-4) and phyloseq (version 1.25.2) packages. Prescriptions of all AMs were grouped in to low (≤ 4), medium (5-13) and high (≥ 14) use. Similarly, the four most common AMs; Amoxicillin (< 1/2-5/>6), Azithromycin (0/1-3/>4), Amoxicillin and enzyme inhibitor (0/1-2 /> 3) and Phenoxymethylpenicillin (0/1/ >2) were also grouped to low, medium and high groups. AM prescriptions used as continuous variable in alpha-diversity and differential abundance analysis. Difference in the alpha-diversity was tested between age groups; gender with *t-test*, and between children's languages; sequence depth groups with ANOVA using Shannon and inverse Simpson index. Difference in the microbial diversity between non-users and recent AM users (1-month, 2-month and 3-month use) were evaluated using the ANCOVA (adjusted for depth). Association of AM prescriptions with microbial diversity was evaluated using linear regression analysis. Prescriptions of all AM and the four most common AMs incorporated in the same model as continuous variable, and adjusted the analysis for gender, age, language and sequencing depth. This was performed for the all participants and separately for boys and girls.

Permutational multivariate analysis of variance test (PERMANOVA) was performed on beta-diversity using the R-package vegan with 999 permutations. The analysis was adjusted for age, gender, language, other AMs and sequence depth. PERMANOVA performed between prescription groups of Low, Medium and High for all AM, and separately for the four most common AMs. Pairwise PERMANOVA was tested between prescription groups (Low-Medium, Low-High and Medium-High) and p-values adjusted with Benjamini-Hochberg method. Differentially abundant bacteria were identified at the OTU level using Negative Binomial GLM fitting and Wald statistics (nbinomWaldTest), implemented in the DESeq2 package (Love et al., 2014). AM prescriptions were used as continuous variable in the abundance analysis. Differentially abundant OTUs at per unit change in the prescriptions of AMs were tested, i.e. a) all AMs, b) Amoxicillin, c) Azithromycin, d) Amoxicillin and enzyme inhibitor, and e) Phenoxymethylpenicillin. OTUs with low counts were filtered out (cut-off 20) in DESeq2 analysis. The analyses were adjusted for gender, age, language, other AMs and depth. The samples without sufficient background information and recent AM users were omitted from the analyses in studies **II** & **III**.

Table 4: Summary of the methods used in the thesis.

Steps	Method	Study	Reference
Saliva DNA extraction	Mechanical & chemical lysis	**I, II, III**	Yuan et al., 2012
PCR amplification	TS-tailed 1-step	**I, II, III**	Study I
	TS-tailed 2-step	**I**	Study I
	NX-tailed 1-step	**I**	Study I
Sequencing	MiSeq	**I**	
	HiSeq	**II, III**	
Taxonomic classification	Miseq_SOP in Mothur pipeline	**I,II, III**	Schloss et al., 2009
Statistical analysis	Kruskal-Wallis test	**I, II**	
	Intraclass correlation coefficients	**I**	
	t-test, ANOVA, ANCOVA, Linear regression	**III**	
	PERMANOVA	**II, III**	
	Differentially abundant bacteria (fitZIG, nbinomWaldTest)	**II, III**	

5. RESULTS

5.1 Reproducibility and repeatability of saliva microbiota profiles (I)

A large-scale study of the saliva microbiota and body size relies on well-developed methodology, exploiting culture-independent NGS technologies. A challenge using NGS was to ensure high reproducibility and repeatability of the method. Thus, one of the study goals was to assess reproducibility and repeatability of saliva microbial profiling methods.

The 16S rRNA amplicons on Illumina sequencing generated 598856 read pairs for 72 samples (4 samples in triplicates x 6 methods) with TS-tailed and NX-tailed amplification, with and without internal index. Control samples (two water samples, nine saliva control samples including 5 replicates) and blank samples using TS-tailed 1S protocol without internal index were sequenced on the Illumina HiSeq platform.

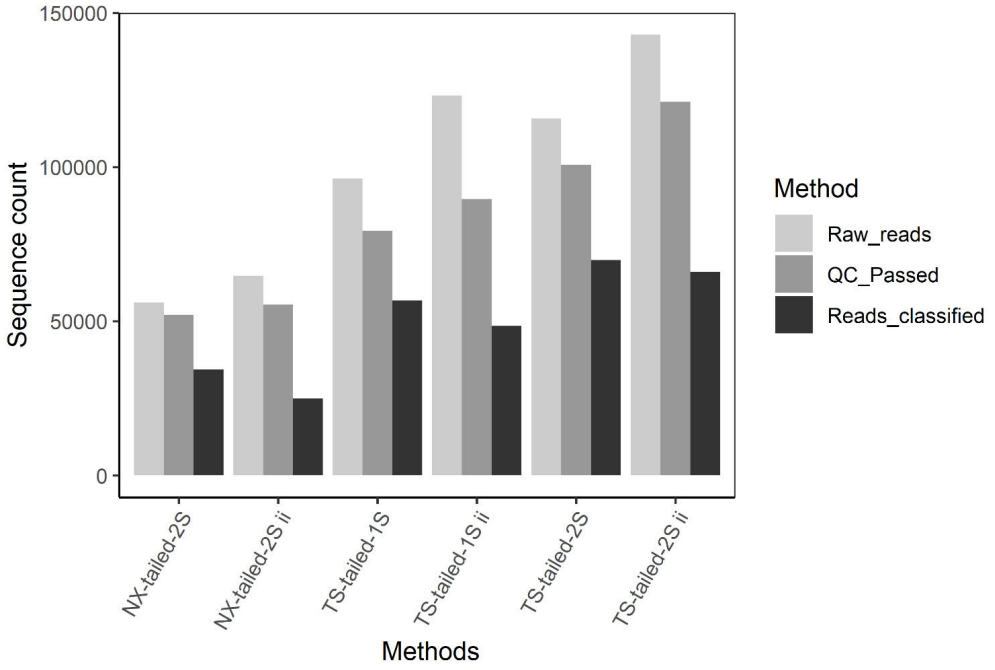

Figure 9: The barplot shows the sequencing statistics of raw reads, passed quality check, and sequences aligned and classified (combined for methods used) in each method sequenced on the MiSeq platform.

Samples sequenced using the TS-tailed 1S and 2S amplification protocols with and without internal index generated higher amounts of raw reads and sequences passed quality checks compared to the NX-tailed protocols (Figure 9). After the quality filtering

and index trimming, the sequences were clustered and assigned to 1086 OTUs. Among all the protocols, the percentage (>58%) of sequences classified in the TS-tailed and NX-tailed were higher in the protocol without internal index. The protocols with internal index showed a drop of 14-23 % OTUs for all the protocols.

5.1.1 Microbial diversity among the protocols

A similar pattern of Shannon diversity was observed in the individual's samples sequenced with different protocols, except in those with low sequence depth (Figure 10). Similar trend was marked with the inverse Simpson index for all the samples. Rarefaction analysis showed that an additional sequencing coverage was required for estimating the microbial richness in saliva, which was resolved by the HiSeq sequencing.

No significant association (KW-test) was found between the alpha-diversity indices and the protocols used. Principle coordinate analysis (PCoA) using Bray-Curtis distance shows that irrespective of methods used, the samples clustered according to the individuals. The relative abundance of saliva microbiota at phylum level was measured. Five phyla were abundant in the samples: Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria and Proteobacteria. Similar patterns of microbial abundance were witnessed for the samples from the same individuals using the different protocols.

5.1.2 Reproducibility and repeatability of the protocols

Intra-class correlation coefficients (ICC) using six metrics included relative abundances of four top abundant phyla. Two alpha-diversity indices showed comparatively better reproducibility and stability with the TS-tailed 2S protocol with and without internal index.

Repeatability of the TS-tailed 1S protocol was tested with nine control samples, two water samples and two blank samples sequenced in HiSeq Illumina platform. The result showed low diversity for the blank samples sequenced and high diversity for the control samples. Comparatively higher alpha-diversity observed for the five replicates of control sample 4 with high sequence depth than the MiSeq sequencing (Figure 11). Bacterial relative abundance of control samples at phylum level show high abundance of the phyla Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria and Fusobacteria.

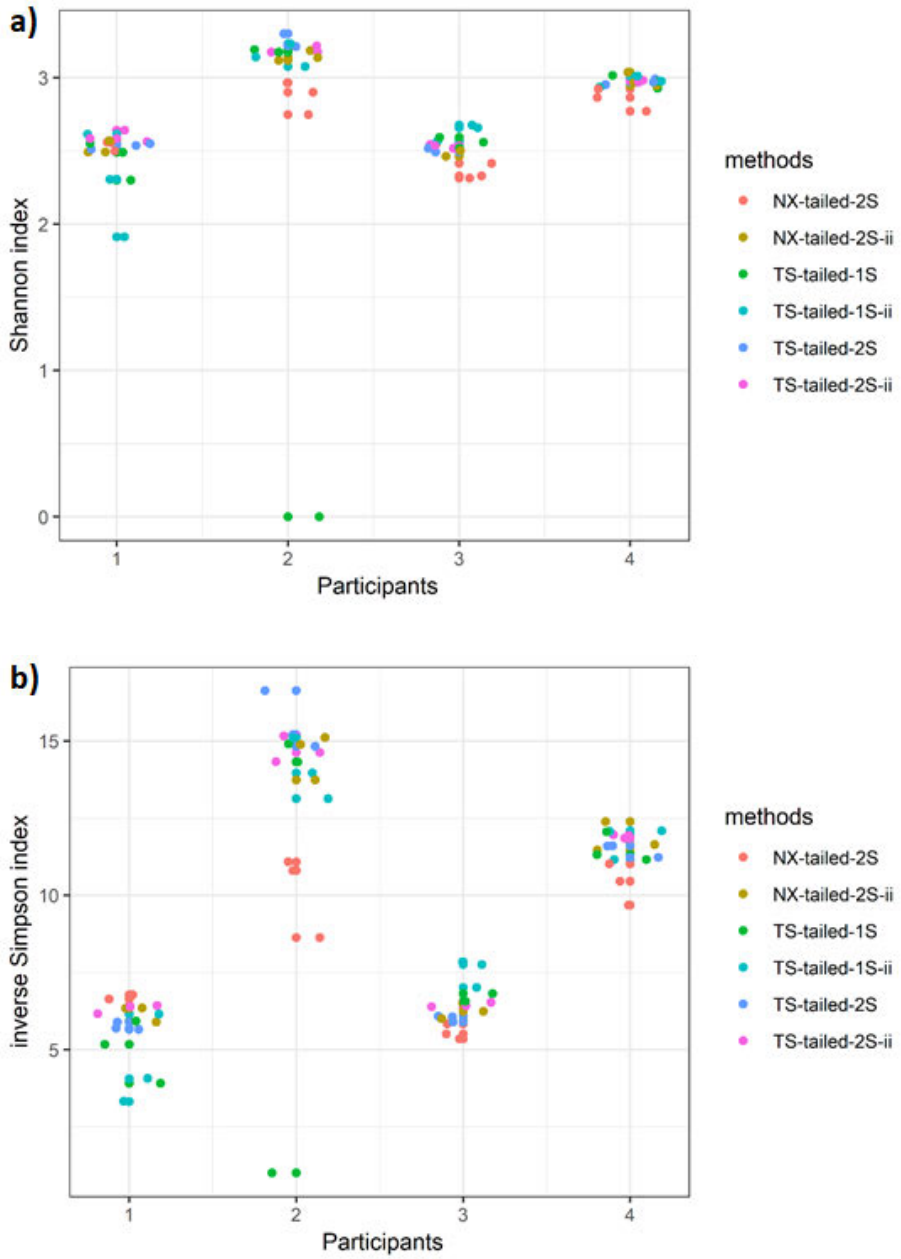

Figure 10: Alpha-diversity using Shannon (a) and Inverse Simpson (b) indices in four individuals' replicates sequenced in different amplification protocols.

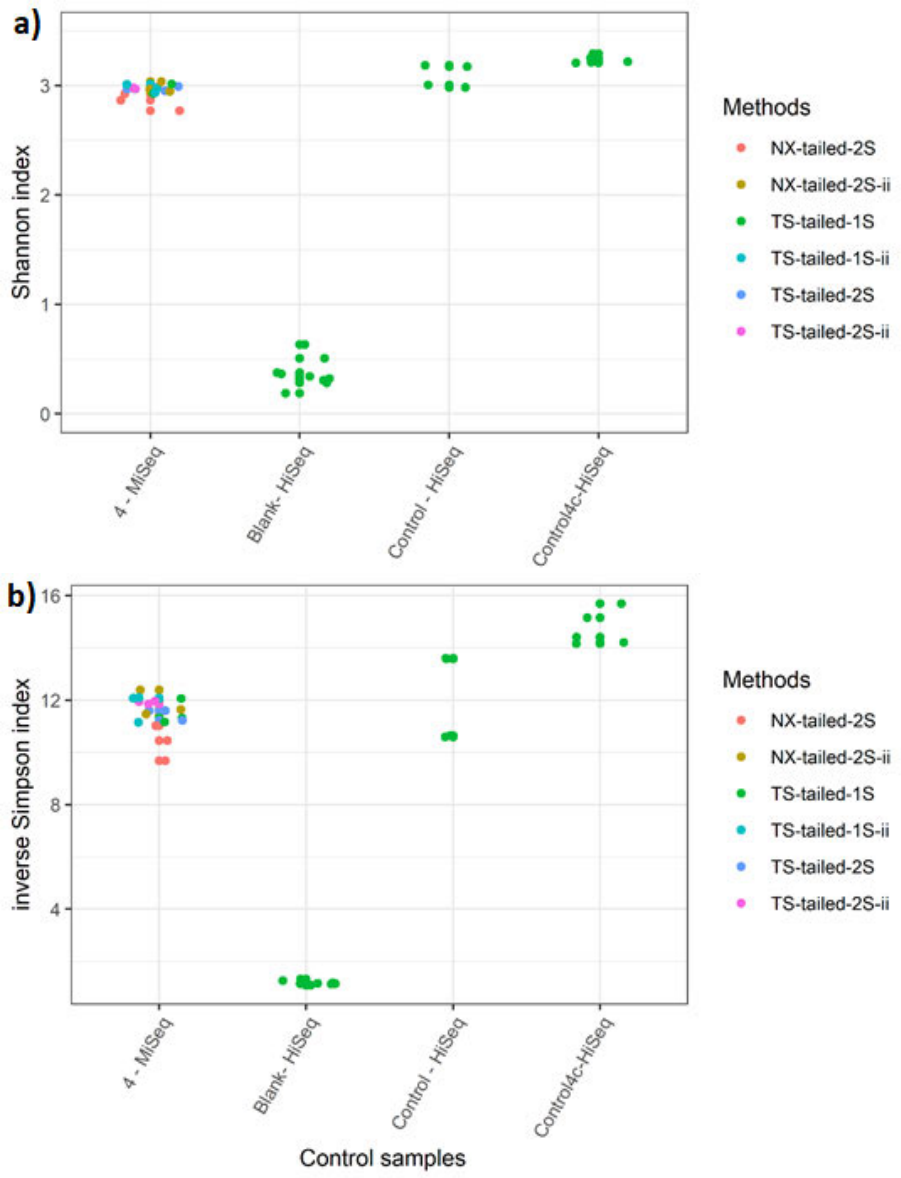

Figure 11: Alpha-diversity using Shannon (a) and Inverse Simpson (b) indices for the participant 4 and its replicates sequenced with MiSeq using different amplification protocols and controls and 4c replicates in Hiseq platform using the TS-tailed-1S method.

5.2 Comparison of the saliva microbiota between individuals with different body sizes (II)

To compare the saliva microbiota between individuals of different body sizes, the participants were categorised into four groups according to age-specific BMI cut offs (Figure 12). Saliva microbial data came from 900 children sequenced with HiSeq

Illumina platform. The participants had a mean age of 11.9 years (S.D = 0.12). Among all, 73.3% of the participants were of normal weight. However, there was a higher proportion of underweight girls than boys (15.9% versus 11.5%) in this study. No significant gender differences in the proportion of overweight and obese children were observed.

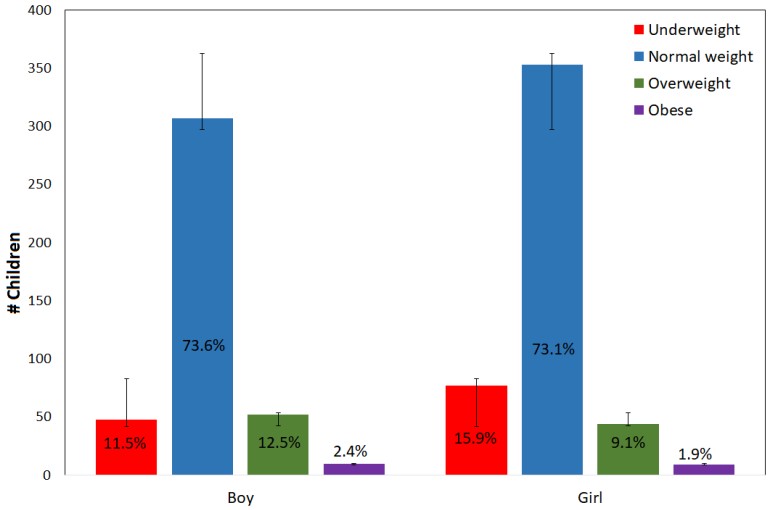

Figure 12: Distribution of the participants (n=900) according to their body size and gender (after removing individuals without metadata or consent or those with recent antimicrobial use).

Approximately 133 million raw read pairs were generated from 16S amplicon sequencing. Rarefaction analysis showed that the sequencing approach and depth (mean 148,508) was sufficient to determine the bacterial richness. On average 48248 assembled sequences per sample were assigned to 6536 OTUs. A few OTUs were mostly assigned to negative control samples and sequences assigned that particular OTU was absent or negligible in Fin-HIT samples.

5.2.1 Alpha- and beta-diversity

No significant alpha-diversity observed between body sizes with all samples using the inverse Simpson index (Figure 13). Alpha-diversity differed significantly between genders. In addition, when the boys and girls were analysed separately, the alpha-diversity of normal weight vs. overweight girls and normal weight vs. obese boys were different. There were statistically significant differences in the microbiota composition among body sizes with all samples, between genders and in gender separated analysis that are resumed in the following chapter 5.2.2.

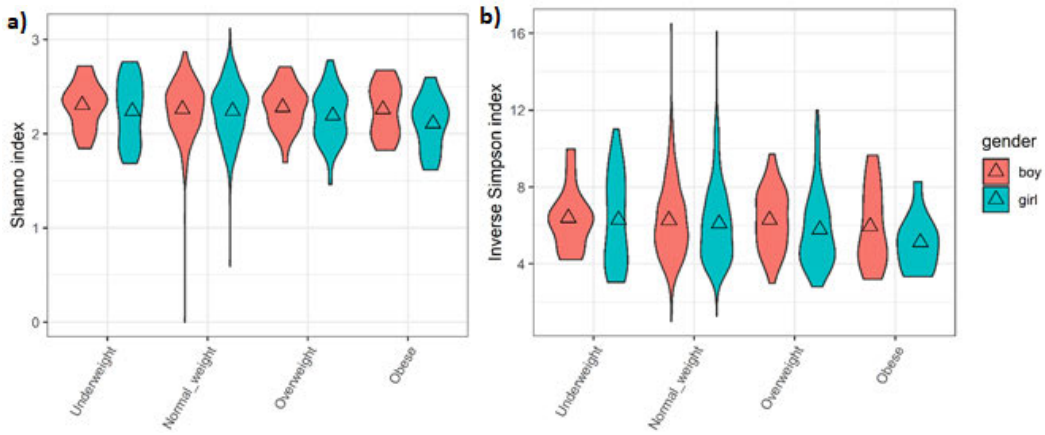

Figure 13: Alpha-diversity using Shannon (a) and inverse Simpson (b) indices for the all participants.

5.2.2 Abundance of microbiota profiles

Core saliva microbiota, i.e. bacteria present in more than 95% of the samples, consists of fourteen bacterial genera including Veillonella, Prevotella, Streptococcus, Neisseria, Selenomonas, Haemophilus, Eubacterium, Porphyromonas, Fusobacterium, Gemella, Campylobacter, Granulicatella, Leptotrichia and Johnsonella. The most abundant bacterial classes in the saliva were Clostridia (39.4%), Bacteroidia (19.3%), Bacilli (11.8%), Betaproteobacteria (7.5%), Gammaproteobacteria (7.4%), Actinobacteria (6.3%) and unclassified bacteria (4.3%) (Figure 14). No significant association of the ratio of Bacteroidetes to Firmicutes was observed to body size. Significant associations between the relative abundance of bacteria at different taxonomic levels and children's body sizes, and among genders were found. A notable finding in the abundance analysis was the many-fold decrease in core commensal saliva bacteria in the overweight and obese children.

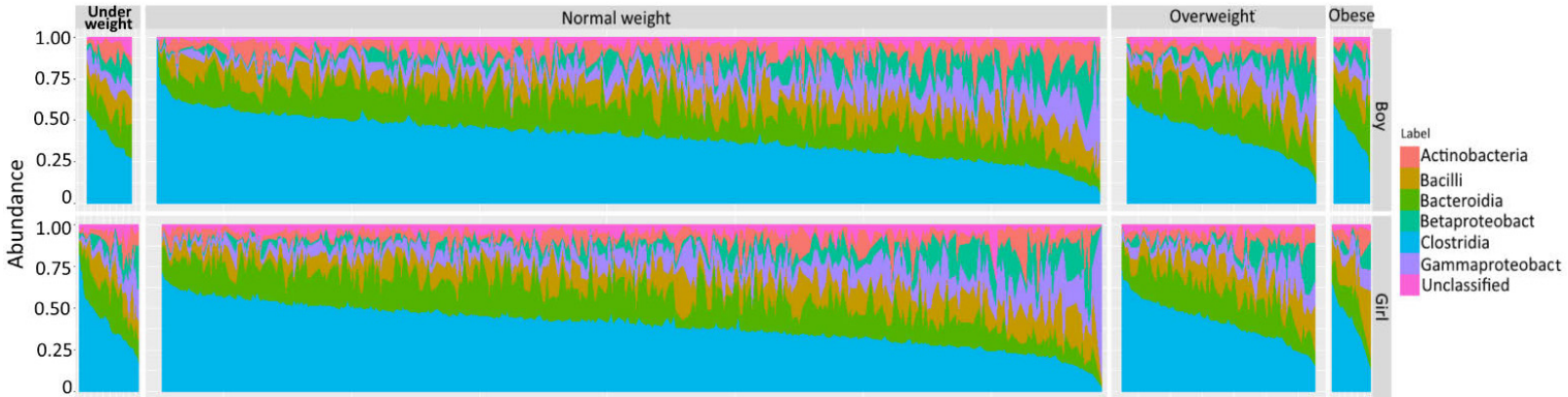

Figure 14: Stacked area plots show the abundant bacterial classes in children belonging to the four body size categories. Bars indicate relative abundances, coloured by taxonomic level class, in boys (top) and girls (bottom). Stacked according to Clostridia.

5.3 The effects of antimicrobials (AM) on children's saliva microbiota (III)

There were 6168 AM treatments prescribed to participants from birth up to 3 months prior to the sampling/participation date. Amoxicillin (n=2698, 43.7%), Azithromycin (n=1542, 25%), Amoxicillin + enzyme inhibitor (n=1147, 18.6%) and Phenoxymethylpenicillin (n=424, 6.9 %) were the most commonly used AMs by the participants. An average use of 7.4 (SD=5.8) AMs per child. Average prescription was 7.9 (S.D=6.0) and 6.9 (S.D=5.7) for any AMs in the boys and girls, respectively.

5.4 Alpha- and beta-diversity

No significant difference in the alpha-diversity was observed between recent AM prescriptions (= the use of AM less than 3 months prior to sampling) and those who are naïve (no prescriptions). No association was observed between prescriptions of all AMs and alpha-diversity using the Shannon index and Inverse Simpson index, as the violin plot shows alike diversity between AM prescription groups**.** Associations for the most common AMs prescriptions were tested and there was a significant association only for Azithromycin with Shannon diversity index.

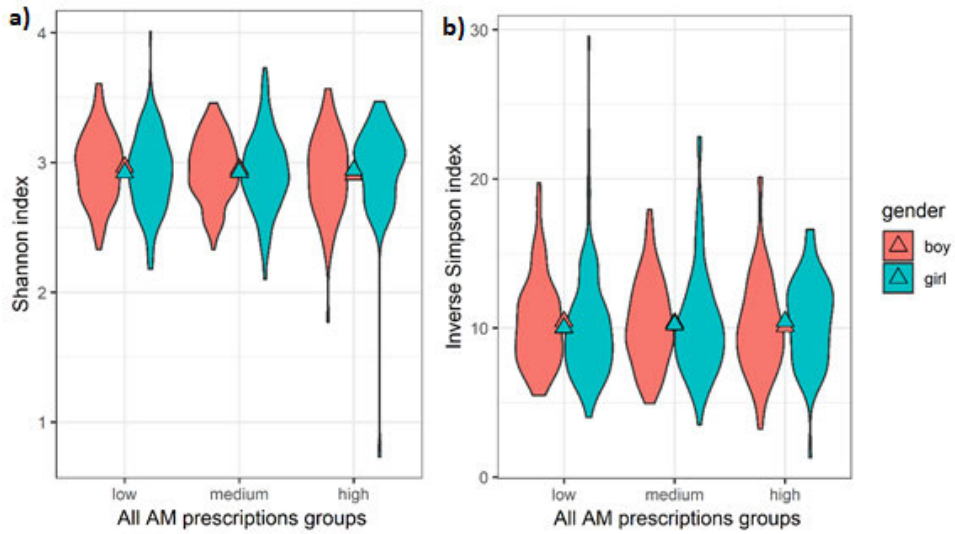

Figure 15: Violin plot shows the distribution of alpha-diversity as measured by a) Shannon and b) inverse Simpson indices for All AM prescription groups separated by gender in children. Triangles inside the plots indicate the mean diversity in the group.

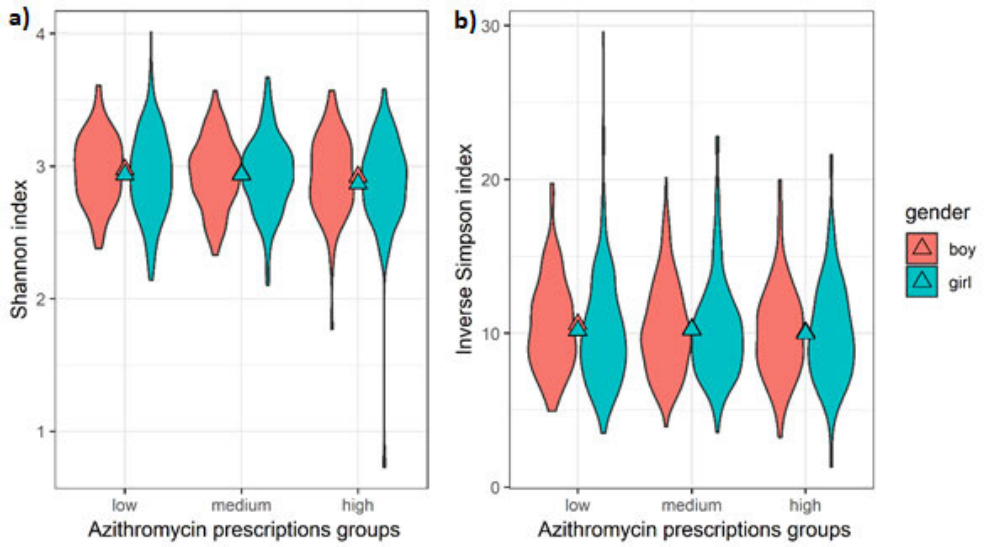

Figure 16: The violin plot shows the distribution of alpha-diversity as measured by a) Shannon and b) inverse Simpson indices for Azithromycin users separated by gender in children. Triangles inside the plots indicate the mean diversity in the group.

In the gender-specific analysis, no association was observed for all AMs, similar result as in all children, with alpha-diversity in both genders (Figure 15). In girls, the association was significant for Azithromycin users as shown by Shannon index (Figure 16). Microbial composition differed between the low, medium and high prescription groups for all AMs and in boys. The microbial composition in Azithromycin users was significantly different in all children, boys, and partially in girls.

5.4.1 Abundance of microbiota profiles

Differentially abundant bacteria per AM prescription at OTU levels were tested. Bacterial taxa *Paludibacter*, Incertae sedis, *Campylobacter*, *Streptococcus*, Rikenellaceae, Prevotellaceae and *Porphyromonas* were differentially abundant in children with different AM prescriptions.

6. DISCUSSION

6.1 Microbiota profiling for large-scale epidemiological studies (I)

The rising interest in the human microbiota and affordable technologies to quantify the microbiota profiles have encouraged researchers to improve the methods to overcome limitations inherent to the sequencing technologies. Several studies have been successfully used Illumina technology for 16S amplicon sequencing for diverse sample types (Bartram et al., 2011; Caporaso et al., 2012; Claesson et al., 2010; Degnan and Ochman, 2012; Fadrosh et al., 2014; Gloor et al., 2010; Kozich et al., 2013; Sinclair et al., 2015; Wu et al., 2018; Xun et al., 2018; Zhou et al., 2011). Through the course of the analyses presented in this study, six Illumina technology based TS-tailed and NX-tailed amplification protocols were used on saliva samples with primers that were modified in-house (Klindworth et al., 2013; Yuan et al., 2012). The primary aim of this study (I) was to simplify the amplification procedure for Illumina sequencing and to make use of barcodes efficiently to run large sample sets on the HiSeq platform.

Saliva harbours numerous and diverse microorganisms shed from various intraoral surfaces. It has been shown that the oral cavity is colonized by 600-700 bacterial species (Chen et al., 2010; Dewhirst et al., 2010). The measured diversity depends on the method used for cellular disruption. Researchers have different opinions on the impact of DNA extraction methods on saliva microbiota profiles (Lazarevic et al., 2013a; Lim et al., 2017). Due to differences in the cell wall composition and structure, the bacterial cells may respond differently to lysis methods. The yield of bacterial diversity with different cell lysis methods, including chemical and mechanical (bead beating) lysis methods have been tested. Mechanical lysis methods give the highest bacterial diversity in 16S amplicon-based studies, especially when the microbial community comprises of gram-positive bacteria, such as faecal samples (Robinson et al., 2016; Salonen et al., 2010; Santiago et al., 2014; Yuan et al., 2012). It has been reported that mechanical lysis (repeated bead-beating) yields greater bacterial diversity in saliva samples due to the rigid bacterial cell walls (Lazarevic et al., 2013a; Sohrabi et al., 2016). A study of mock communities, consisting of seven representative oral bacteria, showed that a method including bead beating was the only protocol which detected all seven species in the community (Abusleme et al., 2014). To reduce the bias that may arise due to the cell lysis step, all samples in the present study were lysed with a protocol including both enzymatic and mechanical disruption of microbial cells using bead beating. The storage and transportation conditions of samples may influence the DNA yields and qualities in microbiome studies (Goodrich et al., 2014a), but these do not have major impact on saliva microbiota (Lim et al., 2017; Nunes et al., 2012).

Four saliva samples in triplicates sequenced in MiSeq platform using different PCR amplification protocols provided higher sequencing depth for TS-tailed protocols and similar microbial diversity for all the protocols. It may be challenging to attain the satisfactory overlap of assembled paired-end sequences with the HiSeq platform. The current read length of 251x2 bp (rapid mode), the V3-V4 region of the 16S rRNA gene is a possible target for sequencing (Mizrahi-Man et al., 2013), however extending the read length to 271x2 bp on the HiSeq platform provided sufficient high quality overlap in the assembled reads.

In MiSeq, though the data obtained with the NX-tailed protocol was low in sequence coverage, this protocol yielded high quality data and discarded a lower number of sequences than TS-tailed protocol (10% vs 18%, respectively). The protocols with internal indices provided a lower percent (<41%) of sequence data for the OTU classification, possibly due to insufficient overlap with additional indexes. Mismatching barcodes may cause loss of data in the microbiota sequencing (Degnan and Ochman, 2012; Sinclair et al., 2015). The indices in NGS have been introduced for multiplexing samples to add a large number of samples in one sequencing experiment (Goodrich et al., 2014a; Kozich et al., 2013). The advantage of error-correcting barcodes has been suggested for reducing the possibility of reads to assign to the wrong sample (Hamady et al., 2008). Repeated use of specific index sequences does not influence the amplicon yield (Goodrich et al., 2014a). A dual indexing protocol can increase the nucleotide diversity by adding random heterogeneity spacer at the start of sequencing reads (Fadrosh et al., 2014). A recent study modified the dual indexing strategy by adding a third Illumina compatible heterogeneous index with variable length to minimise the need for PhiX spike-in (de Muinck et al., 2017). The advantage of dual index with internal index enables sequencing of a large set of samples at low cost, and reduces the polymerase artefacts in high multiplex amplicon sequencing (Peng et al., 2015).

The present study revealed that the numbers of phylotypes or OTUs yielded were comparatively similar in all the methods depending on the sequencing depth, and the rarefaction of the data suggested that bacterial diversity could be attained with higher sequence depth. The microbiota diversity was positively correlated with sequencing depth and was used as co-variable in the two other studies. The differences observed in the sequencing yield may be due to the protocol robustness. There are many reasons that might cause technical variability detected in the microbiota studies; like laboratory protocol, sequencing platform and bioinformatics approach (Salter et al., 2014; Sinha et al., 2015). Nevertheless, in the present study all protocols delivered comparatively similar microbiota profile for the saliva triplicates. Technical challenges may arise in 16S rRNA amplicon sequencing due to the PCR primer selection, PCR template concentration and amplification conditions and sequencing. The importance of investigating technical replicates to validate the reproducibility of microbiota methods has been discussed recently (Wen et al., 2017). The average alpha-diversity for the four

samples from different protocols provided comparatively similar diversity with the exception of samples with low amount of sequences. With the mixed-effect model–based Intra Correlation Coefficient analysis (Sinha et al., 2016), sequencing protocols using TS-tailed 2S protocol with and without internal index performed better than the other protocols tested.

The repeatability of the TS-tailed 1S method without internal index was tested on HiSeq platform. Nine samples sequenced on HiSeq yielded high sequence depth (48K-398K) reads. The reproducibility can be increased by increasing sequencing depth. Our samples had comparatively high alpha-diversity for the data produced with HiSeq sequencing and low variation among them. Alpha-diversity was similar for the sample 4 sequenced on both MiSeq with different protocols and HiSeq platform with the TS-tailed 1S protocol. The major limitation of this study is the small number of samples for testing in each method, however, the number of samples and the sequencing depth was seen sufficient to recommend the method that should or should not be used in large-scale microbiota studies.

6.2 Comparison of saliva microbiota in children with different BMI (II)

The purpose of this large-scale study was to characterize the saliva microbiota in a population of children with different BMI, which had not been explored previously. Different saliva microbiota indices and profiles in underweight, overweight, obese and all overweight (+obese) was compared to normal weight children. To our knowledge, this study with 900 participants is the first large-scale study that explores the association of the saliva microbiota and body sizes in children. The sample size (483 girls and 417 boys) ensured statistical power to detect differences in microbiota between body sizes, and genders. The amplicon methods (TS-tailed 1S without internal index) used ensured large amount of sequence data (mean 148,508 read pairs).

The microbiota assists the host in resisting invasion of pathogens, and its biodiversity is known to be associated with health. It has been suggested that gut microbiota composition in early life predicts weight development in later childhood, and disruption of the original microbiota may have health consequences in later stages of life (Korpela et al., 2016). A decreased gut microbiota diversity has been linked to obesity (Turnbaugh et al., 2009) and many systemic and non-systemic diseases (Wang et al., 2017). Similar associations between altered oral microbiota and health have also been reported (Acharya et al., 2017a; Belstrom et al., 2016; Xun et al., 2018). A recent study in adults showed that obese groups have distinct saliva microbiota profiles compared to normal weight adults (Wu et al., 2018). In the present study, the saliva microbiota was associated with children's BMI. The differences observed in the saliva microbiota were gender-specific, indicating the microbiota-body size association differs between boys and girls. The finding is in line with the gut microbiota where distinctive bacteria were

associated with men and women which may be inclined by the severity of obesity (Haro et al., 2016). The gender differences in the present study may be induced by endocrine and hormonal factors (Zaura and Ten Cate, 2015) as the participants are about to enter puberty. Beta-diversity analysis suggests that the bacterial composition in saliva was significantly different between the normal weight and obese participants, but in girls only. Significant gender-differences in bacterial composition were also observed. The differences in the alpha-diversity between body sizes and gender were also reflected in the beta-diversity analysis. A study on the upper digestive tract microbiome of adults showed that beta-diversity was associated with a four-fold difference in obesity (Lin et al., 2015).

Among the present cohort, microbial abundance differed between underweight, normal weight, overweight and obese children. A key finding was a many-fold decrease in the relative abundance of core saliva bacteria in children of all body sizes. The genus *Prevotella* associated with BMI may potentially be a novel marker in saliva to identify the children at risk of gaining excess weight. To support this hypothesis, a study on gut microbiome of adolescents (aged 13-16 year) has shown that *Bacteroides* and *Prevotella* were associated with BMI and positively correlated with triglycerides, total cholesterol, and high-sensitive C-reactive protein (Hu et al., 2015). However, the BMI-associated bacteria of saliva in this study were different from those found in the gut (Human Microbiome Project Consortium et al., 2012; John and Mullin, 2016), confirming the differences between saliva and gut microbiota diversity and composition in different obesity states. Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Candidate division TM7, and Fusobacteria were the six dominant (>3%) phyla in the saliva of children, and these findings are in line with earlier studies (Lazarevic et al., 2012). The abundant bacterial taxa in the children's saliva were *Veillonella, Prevotella, Streptococcus, Selenomonas, Neisseria, Haemophilus*, Micrococcineae, *Gemella*, and *Neisseria*. Individual genetics and lifestyle, geography, and variations in the lysis procedure and PCR amplification may be the reason of differences in the taxa abundances (Lazarevic et al., 2011).

The strengths of the present study include a large sample size and a homogeneous age-group of children who do not normally suffer from periodontitis and other chronic diseases (Alabdulkarim et al., 2005; Sarlati et al., 2008). The limitation of this study is the lack of oral health status, eating habits and pubertal status of the children, which are potential confounders. In Finland, public oral health care services are freely accessible to children under 18 years, most of the children and adolescents have good oral health and declining trends in caries occurrence rate has been seen (Mattila et al., 2016). Other factors that may influence the results such as dieting, smoking and alcohol consumption are unlikely in this age group. However, these findings from children may not be generalizable to adults and elderly populations. As a part of the educational system in Finland, children receive healthy lunches and snacks at school, for which we assume

that a major part of the participants' diet is similar and did not take into account the potential dietary confounding in this study. An on-going study have not identified associations between eating habits and saliva microbiota in this cohort (personal communication Viljakainen et al), supporting this assumption. The recently developed molecular methods help researchers to expand the knowledge of the composition and function of the oral microbiota in health and disease (Gao et al., 2018). It has been shown that the saliva microbiota is more resilient and stable despite the changes in diet and oral hygiene (Stahringer et al., 2012). Thus, the saliva-based health monitoring using microbial biomarkers may be considered a feasible, simple, easy to collect with high compliance for all age groups and economical alternative compared to faecal samples.

6.3 The effects of lifetime antimicrobials on children's saliva microbiota (III)

The purpose of the study was to describe the association between saliva microbiota and lifetime use of antimicrobials in children, which has not been explored previously. Saliva microbiota diversity indices and profiles were analysed in AM users and non-users. The microbiota diversity in the Azithromycin user groups differed in a gender-specific way and was more profound in girls than boys. The findings are in line with a saliva microbiota study where diversity and composition were altered by short-term azithromycin use (Cabral et al., 2017). Association between saliva microbiota and recent AM use within three months prior to sampling was not identified. Hence, AM use in recent years might not influence the saliva microbiota in children in the long-term. The salivary community seems to be more stable and resilient and less susceptible to dysbiosis resulting from of recent antimicrobial perturbations, but longitudinal studies are needed from the infancy to the adulthood to study the long-term resilience, stability and consequences.

The microbial composition was significantly different between all AMs combined user groups and in Amoxicillin and Azithromycin in all children, and separately in boys. The results are in line with a recent study investigating children's gut microbiota, in which it was reported that macrolides impact the microbiota composition (Korpela et al., 2016). AMs are commonly used drugs to treat the bacterial infections, which ultimately result in the reduction of pathogenic and commensal bacteria in humans and animals (Zaffiri et al., 2012). The immediate effect of AMs on the gut microbiota is higher than any other factors (Dethlefsen and Relman, 2011; Modi et al., 2014). AM use in childhood is associated with noticeable changes in the gut microbiota composition, which persist for over six months (Korpela et al., 2016). Also, a short-term effect of AMs has been found on the saliva microbiota (Abeles et al., 2015; Zaura et al., 2015). Paediatric populations are commonly exposed to infections and AM use which can disrupt the natural microbiome from very early stage of life (Taggart and Bergstrom, 2014; Virta, 2012). No significant difference in the association of the saliva microbiota and lifetime AM use

was observed in the sensitivity analysis, after excluding the AM use in the first three years in the participants early life.

The present study did not investigate the disruption and re-establishments of microbiota with the effect of one single treatment, but the effect of several repeated treatments. An increased use of AM puts humans at increased risk for being a carrier of antibiotic-resistant bacteria (Costelloe et al., 2010). The majority of studies on antibiotics are performed in adults, or have analysed short-term effects on the microbiota of children. Up to date, the present study is the first to explore the effect of lifetime (~ 12 year) AM use on the saliva microbiota. The strength of the study includes validated national registers. The register keeps the records of not only the use of the drug, but also the antimicrobial drug purchases which is a strong indicator of AM intake. The diagnoses were not recorded in the drug purchase register; however, similar pattern of AM use have been observed in boys and girls.

Men and women differ in their microbiome composition (Levy and Solt, 2018). Study on the characteristics of gender-specific microbiome was first reported in 2011 (De Cárcer et al., 2011). Flak et al coined the term 'microgenderome' in 2013 which refers to the interaction between microbiota, sex hormones and immune system (Markle et al., 2013). The gender-specific saliva microbiome and metabolome profiles have been associated with salivary pH and dietary protein intake (Zaura et al., 2017). Some of these differences observed between genders are consequences of hormones. Twin studies demonstrated that the microbial composition of opposite sex twins differed when they attained puberty compared to same sex twins (Wallis et al.; Yatsunenko et al., 2012). The gender-specific fat distribution associated with the differences in sex hormone levels (Haro et al., 2016). There is a gender-specific association between the early antibiotic exposure and body size development (Ajslev et al., 2011). The biological explanation for this gender specificity remains unexplored, but could be related to microbiota–host interactions (García-Gómez et al., 2013; Markle et al., 2013).

As shown in study **II**, there is an association between the saliva microbiota and body size in children. The use of antimicrobials, especially macrolides, in early life was positively correlated between overall lifetime antibiotic use and BMI, as well as an increased risk of asthma later in childhood (Korpela et al., 2016). Antibiotics have been linked to gut microbiota dysbiosis, which may lead to obesity, however, the mechanisms through which AMs modulate the weight development is unclear (Leong et al., 2018). Several hypotheses have been proposed: altered energy extraction from indigestible polysaccharides; an altered number of bacteria that are metabolically protective against obesity, metabolic signalling, hepatic lipogenesis, and intestinal defence and immunity (Leong et al., 2018). A similar human microbiota study has been reported the gender-dependent gut microbiota in relation to BMI (Kim et al., 2019). The factors affecting the gender differences in gut microbiota may be due to the interaction with sex hormones.

Alpha-diversity differed significantly between males and females after puberty (Yurkovetskiy et al., 2013). In the present study, the participants were children of 11.7 years of age (mean), and some participants might have reached puberty. Findings in the present study suggest that young children might be vulnerable to weight gain due to extensive use of AMs. The frequent use of AMs cause rise in antibiotic-resistant bacteria in children, which can make therapeutic treatments challenging in the future.

7. CONCLUSIONS AND FUTURE PROSPECTS

Large-scale epidemiological studies on microbiota are feasible with the recent advancements in the NGS technologies and culture-independent molecular techniques. The challenge in obtaining reproducible microbiota profiles using NGS was addressed in this thesis. All the different indexing schemes tested produced microbiota profiles with high reproducibility. The TS-tailed-1S dual index protocol was preferred and used in the other two studies of this thesis since it delivers reproducible and repeatable profiles on the HiSeq platform and is economic and less labour intensive.

The thesis shows that saliva microbiota is associated with BMI and lifetime antimicrobial use and is gender-specific. The prominent finding of this study was the many fold decrease in the core commensal saliva bacteria in overweight and obese children compared to normal weighted children. The BMI and gender-specific saliva microbiota profiles open new possibilities to study the potential roles of microbiota in weight management. In addition, there is concern over the consequences of high lifetime AM use, which substantially decrease bacterial richness and composition in children. The use of azithromycin had a significant effect on the saliva microbiota. The exposure to antimicrobials in the first decade of life may cause the disruption of natural microbiota, which may have long-term health consequences in later stages of life. Thus, emphasizing the importance of microbiota in developing new therapeutic strategies at early life and childhood and, to sensibly limit the use of AMs. Saliva microbiota was associated with BMI and antimicrobials and it was gender dependent. The gender specific saliva microbiota profiles may be due the children's pubertal stage, where diversity differs after the puberty in humans.

Saliva microbiota is more resilient than gut microbiota when exposed to antibiotics and stable for several years even when changes in diet and oral hygiene occur. Thus, the saliva-based screening of microbial biomarkers, in health surveillance and the associations with oral and general health status may be considered a feasible, simple, easy to collect with high compliance for all age groups and economical alternative. However, further research on the metabolic and functional potentials of the saliva microbiota is needed to fully understand the saliva microbiota – host relationship. The rise in AM use and increased antimicrobial resistance (AMR) is a major threat to human. To effectively tackle this problem, whole microbiota needs to be investigated to discover the range of different AM resistant genes in bacteria. With metagenomics sequencing, we can explore the functional details of bacteria in saliva and antibiotic resistance genes in bacteria. Because most of the bacteria are uncultivable in the laboratory, metagenomics approaches need to be used to explore the functional potential of saliva microbiota. It also helps to explore the mobile genetic elements that cause the spreading of AMR genes across saliva bacteria.

TIIVISTELMÄ

Ihmisen iholla ja limakalvoilla elävä normaalimikrobisto auttaa ylläpitämään terveyttä ja torjumaan sairauksia. Elämäntapatekijät, kuten ruokavalio ja fyysinen aktiivisuus sekä ympäristö ja altistus antibiooteille todennäköisesti muokkaavat bakteeristoa keskeisellä tavalla. Suolistobakteerit näyttävät olevan yhteydessä ylipainoon ja lihavuuteen, mutta syljen bakteeriston ja painoindeksin (BMI) välisiä yhteyksiä on vielä toistaiseksi tutkittu niukasti. Lihavuus on maailmanlaajuinen epidemia, ja vuonna 2016 ylipainoisia ja lihavia oli jo 1,9 miljardia. Lapsuudenaikainen ylipaino ja lihavuus todennäköisesti jatkuu aikuisuuteen ja on yhteydessä suurentuneeseen sairausriskiin varhaisemmalla iällä. Siksi bakteerien ja painon välinen yhteys on tärkeää selvittää.

Antibioottien käyttö ja samalla antibioottiresistenssi on lisääntynyt. Resistenssi johtaa siihen, että monen sairauden hoitomahdollisuudet kutistuvat. Altistuminen antimikrobilääkkeille (AM-lääkkeet) vaikuttaa suoliston bakteeriston monimuotoisuuteen ja koostumukseen, mutta niiden vaikutuksista syljen bakteeristoon tiedetään toistaiseksi vähän. Lapset käyttävät AM-lääkkeitä usein, joten syljen bakteeriston ja käytön välisiä yhteyksiä on tärkeää tutkia. Tämän väitöskirjan tavoite oli kehittää kustannustehokas menetelmä, jolla syljen bakteeristoprofiili voidaan määrittää laajoissa väestöaineistoissa, sekä lisäksi käyttää tätä uutta menetelmää syljen bakteeriston ja painon sekä elinaikaisen AM-lääkkeiden käytön välisten yhteyksien määrittämiseksi lapsilla.

Tulokset osoittivat, että kaikkien testattujen 16S rRNA -geenin monistukseen ja sekvensointiin käytettyjen menetelmien tuottamat syljen bakteeristoprofiilit olivat keskenään samankaltaisia, ts. mikään menetelmistä ei ollut selvästi muita parempi. Syljen bakteeristoprofiilit olivat sukupuolelle ominaisia alfa- ja betadiversiteetin sekä tiettyjen bakteerien suhteellisen runsauden suhteen. Merkittävä löydös oli ylipainoisilla ja lihavilla lapsilla näkynyt ydinbakteeriston väheneminen. Elinaikaisen AM-lääkealtistuksen ja syljen bakteeriston analyysi osoitti, että atsitromysiinin käyttö oli yhteydessä alentuneeseen alfadiversiteettiin kaikilla lapsilla sekä erikseen tytöillä. Syljen bakteeriston koostumus erosi merkitsevästi matalan, keskitason ja korkean AM-lääkkeiden käytön ryhmien välillä kaikilla lapsilla. Tarkasteltaessa eri antibiootteja erikseen atsitromysiinin käytön suhteen löydettiin samanlainen suuntaus, mutta amoksisilliinin kohdalla bakteeriston koostumus erosi merkitsevästi ainoastaan pojilla.

Tämä väitöskirjatyö esittää, että syljen bakteeristo on merkitsevästi yhteydessä painoon, AM-lääkkeiden käyttöön ja sukupuoleen suomalaislapsilla. Syljen bakteeriston profiloinnilla on mahdollista kehittää uusia tapoja tutkia bakteerien mahdollista roolia lasten painonkehityksessä. Vaikutukset normaalibakteeristoon tulisi ottaa huomioon myös kehitettäessä uusia hoitomenetelmiä, jotta AM-lääkkeiden käyttöä saataisiin rajoitettua. Aiemmat tutkimukset ovat osoittaneet, että syljen bakteeristo on vastustuskykyisempi ja vakaampi antibiootteja vastaan kuin suoliston bakteeristo, joten

mikrobimarkkereiden seulonta syljestä ulostenäytteen sijaan on helppo, taloudellinen ja kaikille ikäryhmille soveltuva vaihtoehto käytettäväksi terveydenhuollossa. Bakteeriston metabolisia ja toiminnallisia piirteitä on kuitenkin tutkittava lisää, jotta syljen bakteerien ja isännän välisiä vuorovaikutuksia ja niiden merkitystä terveydelle voidaan ymmärtää paremmin.

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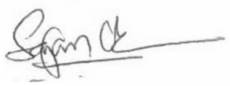

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